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Expression and functional analysis of the development of mesencephalic dopaminergic neurons in the chicken embryo

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Contents

1 Abstract

Dopamine (DA)-synthesizing neurons constitute a prominent population in the mammalian brain as they are involved in the control and modulation of motor, cognitive, rewarding and neuroendocrine functions. The best studied DA population are the mesencephalic DA (mesDA) neurons, since the degeneration of these neurons leads to Parkinson´s Disease in humans. The mesDA neurons of the mouse arise from the ventral midline of the midbrain and caudal forebrain during embryonic development, and different factors control their induction, specification, proliferation and differentiation. To elucidate conserved pathways for the generation of the mesDA population in higher vertebrates, I performed a spatiotemporal expression analysis of the chicken orthologues for the mouse mesDA marker genes *Aldh1a1*, *Nr4a2*, *Pitx3*, *Lmx1b* and *Th* during early chicken embryonic development. I found striking differences in the expression patterns of *Aldh1a1* and *Pitx3* between the two species, suggesting that the corresponding proteins acquired distinct functions in birds and mammals during evolution. In addition, I provide evidence that a subpopulation of the mesDA precursors are born in the diencephalon of the chicken embryo, probably reflecting the phylogenetic origin of this neuronal population.

For the mouse embryo it has been shown that the secreted glycoprotein Wnt1 regulates a genetic network that is required early for the establishment of the mesDA progenitor domain and late for the terminal differentiation of mesDA neurons during embryonic development. To investigate whether Wnt signaling is also required in the development of mesDA neurons in the chicken embryo, first an expression analysis was performed for several *Wnt* genes during embryonic development to determine whether these *Wnts*, and *Wnt1* in particular, are expressed in a similar pattern in the chicken as in the mouse embryo. Here I found that not *Wnt1* but *Wnt9a* is expressed in the ventral cephalic flexure of the chicken embryo. Second, overexpression of Wnt9a in the chicken embryo was used to assess whether the Wnt-controlled induction of mesDA neurons is a conserved mechanism between chicken and mouse. Indeed, the ectopic expression of Wnt9a led to an induction of several mesDA marker genes in the ventral part of the fore- and midbrain. In addition, *Shh* was identified as a downstream target of Wnt9a and the mesDA marker gene *Pitx3* as a very likely transcriptional target of Shh in the chicken embryo.

2 Introduction

The generation of distinct neuronal cell types in appropriate numbers and at precise positions is a fundamental process during the development of the central nervous system (CNS). CNS development begins with the induction of neural cells in the ectoderm of the early embryo by a combination of signals that emanate from the gastrula organizer. These neuroectodermal cells form the neural plate, which will subsequently fold up and fuse at the dorsal apex to build the neural tube. Due to patterning events along the anterior-posterior (A/P) axis the anterior end of the neural tube will then develop into the three brain vesicles: the prosencephalon or forebrain, the mesencephalon or midbrain and the rhombencephalon or hindbrain (Fig. 1A). At the same time the dorso-ventral (D/V) axis of the neural tube is subdivided from ventral to dorsal into the floor plate (FP), the basal plate (BP), the alar plate (AP) and the roof plate (RP) (Fig. 1B). After the early patterning events, secondary organizers are established, e.g. the isthmic organizer at the boundary between the midbrain and hindbrain (MHB) or the zona limitans intrathalamica (ZLI) in the forebrain, which are responsible for a more regional A/P patterning. In addition, signals from the FP and RP provide information in the D/V aspect. Thus, the patterning during early embryonic development leads to a Cartesian grid, which provides positional identity to each of the neuronal precursor cells in the neural tube. The result of these regional identities is the specification of distinct subtypes of neurons in distinctive areas in the developing brain. One of these neuronal populations comprises the dopaminergic (DA) neurons in the midbrain. These neurons have been subject of extensive research in the last decades because of their implication in different neurological diseases, such as Parkinson's disease (PD). To find factors which are important for the induction and/or specification of the DA neurons in the midbrain, loss- and gain-of-function studies have been done and are still required. Only recently, evolutionary studies have come into focus, because they support the revelation of conserved molecular mechanisms in the development of DA neurons in the midbrain.

Neurons that synthesize the neurotransmitter dopamine are found throughout the brain, where they exert a variety of functions on the basis of their wide connectivity within distinct pathways (reviewed by Prakash and Wurst, 2006). The most prominent are the DA

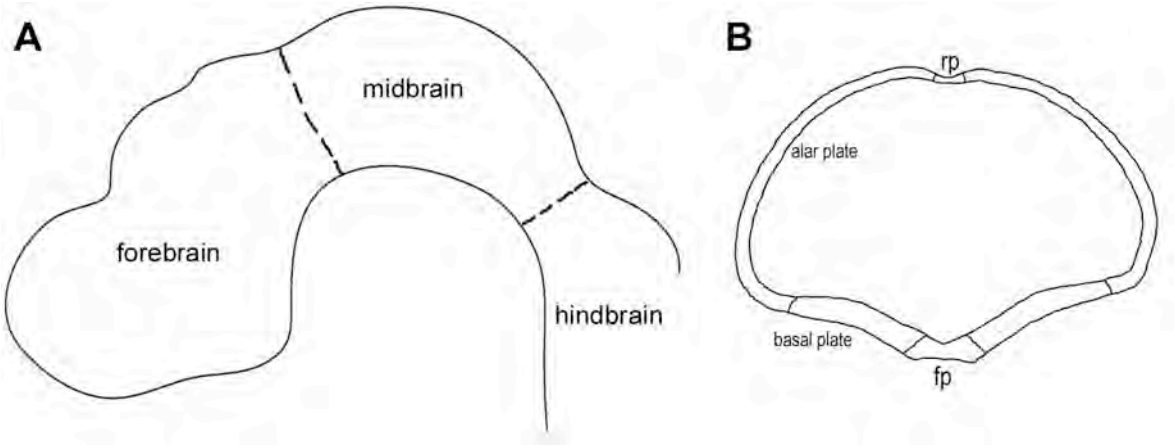

Figure 1: Schematic illustration of a sagittal section (A) and a coronal section of the midbrain (B) of a chicken embryo at E2.5. The neural tube is subdivided in the A/P axis into forebrain, midbrain and hindbrain (A) and in the D/V axis into the floor plate, basal plate, alar plate and roof plate (B). Abbreviations: fp, floor plate; rp, roof plate.

neuronal populations of the ventral midbrain comprising the A8 (Retrorubral field, RrF), A9 (Substantia nigra, SN) and A10 (Ventral tegmental area, VTA) groups in mammals (Dahlstrom and Fuxe, 1964; Hökfelt et al., 1984; Fig. 2). Neurons in the VTA give rise to the mesocorticolimbic system, whose projections terminate in the frontal cortex and the ventral aspect of the striatum. The mesocorticolimbic system is involved in the control and modulation of cognitive and affective behaviors. Dysfunction of this system has been linked to the pathogenesis of drug addiction, depression and schizophrenia in humans (reviewed in Prakash and Wurst, 2006). The SN DA neurons innervate the dorsal part of the striatum, located in the ventral forebrain, and integrate into a circuit that controls voluntary movements and body posture. Degeneration of these neurons leads to Parkinson's Disease (PD) in humans (Klockgether, 2004). These pathologies are the reason of the ventral mesencephalic dopaminergic (mesDA) cell population being a subject of intensive biomedical research in the last years.

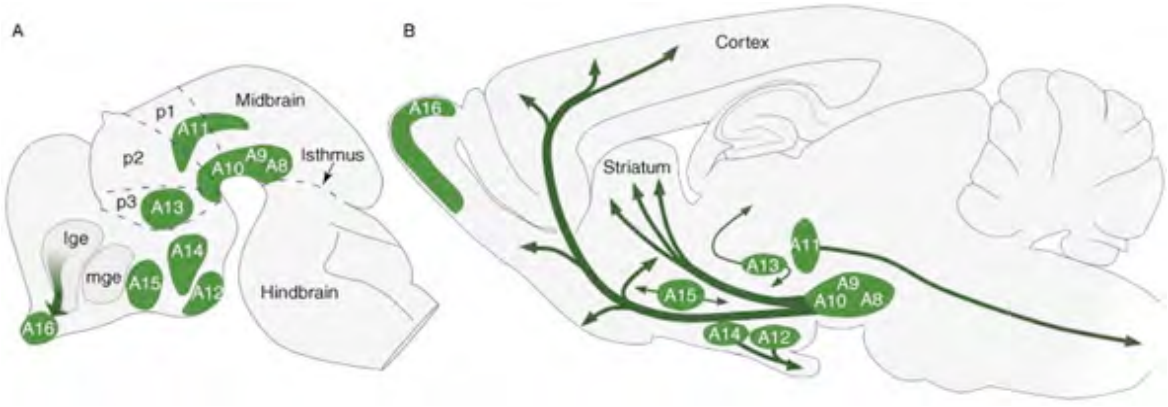

Figure 2: Distribution of DA neuron cell groups in the developing (a) and adult (b) rodent brain. The DA neurons in the mammalian brain are localized in nine distinctive cell groups, distributed from the mesencephalon to the olfactory bulb, as illustrated schematically, in a sagittal view, in **(A)** the developing and **(B)** the adult rat brain. The numbering of the cell groups, from A8 to A16, was introduced in the classic study of Dahlström and Fuxe in 1964, and is still valid at present. In **(B)** the principal projections of the DA cell groups are illustrated by arrows. Abbreviations: lge, lateral ganglionic eminence; mge, medial ganglionic eminence; p1–p3, prosomeres 1–3. Modified from Björklund and Dunnett (2007).

2.1 Development of the mesDA system in vertebrates – the anamniote-amniote transition

In the transition between anamniotes (fish and amphibians) and amniotes (reptiles, birds and mammals), the mesDA system seems to have evolved by adding cells in the A8-A10 complex and thus expanding laterally and caudally towards mesencephalic territories (Smeets and Gonzalez, 2000). On the basis of immunohistochemical, functional and developmental data, the current view on the organization of the connectivity of the basal ganglia is that a common pattern exists among all vertebrates. For a better understanding of the origin of the mesDA neurons in higher vertebrates, developmental studies are ongoing in both anamniotes and amniotes. Until recently, it was unclear whether anamniotes like teleost fish or amphibians possess an equivalent of the human SN and VTA. The dopaminergic neurons projecting to the basal forebrain of amphibians constitute a continuous field along the rostrocaudal axis of the diencephalic-mesencephalic basal plate, whereas the DA population in bony fish is restricted to the diencephalon (Fig. 3). Since the ventromedially located A10 cell group as well as the dorsolaterally extending A9 cell

group in mammals are considered classically to be located in the mesencephalic tegmentum, the apparent differences in the topography of mesDA neurons in amniotes and amphibians have traditionally constituted a strong argument against their homology (Fig.3). Thus, it has been concluded that the presence of basal ganglia structures equivalent to the amniotic system is missing in lower vertebrates. This issue has been reconsidered now by the demonstration that the dopaminergic cell population which is located in a posterior diencephalic position, the so-called posterior tuberculum, in the zebrafish could be the homolog of the amniotic mesDA system (Rink and Wullimann, 2002a; Fig.3).

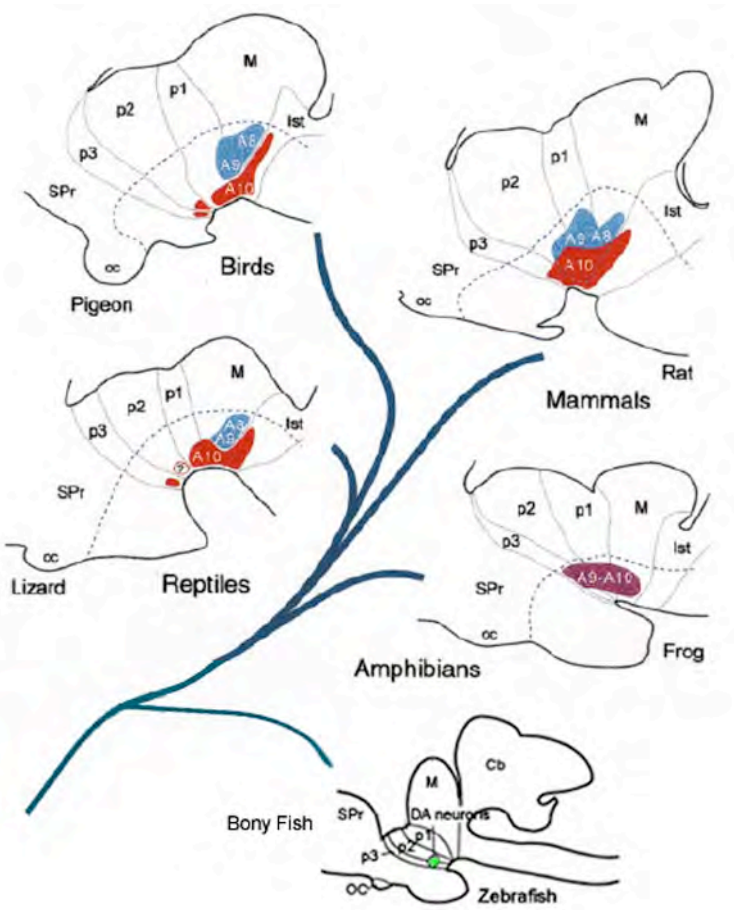

Figure 3: Localization of the DA A8-A10 cell groups in vertebrates. The localization of the DA cells of the VTA (A10), SN (A9) and rRF (A8), with special reference to neuromeric boundaries, is depicted in schematic drawings of midsagittal sections of representative brains belonging to five different classes of vertebrates. Solid lines mark the segmental boundaries. In all vertebrates, except the zebrafish, a continuous paramedian population of DA cells extends across several di- and mesencephalic segments. In early anamniotes (bony fish), the DA population is restricted to the diencephalon, whereas in later anamniotes (amphibians) this cell population already extends into the mesencephalon. Anterior is left. Abbreviations: Cb, cerebellum; M, mesencephalon; p, prosomere; SPr, secondary prosencephalon; Ist, isthmus; oc, optic chiasm. Modified from Marin et al. (1998).

Based on their projections and their function, it was suggested that the diencephalic DA neurons in the zebrafish represent the teleostean dopaminergic system ascending to the striatum (Rink and Wullimann, 2002b). However, with the help of the prosomeric model established by Puelles and Rubenstein (2003) it has been demonstrated that also in amniotes the mesDA cells are not restricted to the mesencephalic tegmentum but extend into the diencephalon (Marin et al., 1998; Puelles and Verney, 1998). As stem reptiles were ancestral to all present amniotic forms and derived from anamniote amphibian stock the question is now whether the diencephalic DA neurons are the phylogenetic precursors of the later in evolution appearing mesDA neurons. The transition from anamniotes (aquatic) to amniotes (terrestrial) and the accompanying enhanced need for motor coordination probably have led to an expansion of the basal ganglia into the mesencephalon and subsequently to a specification of the individual DA cell groups.

In this work, I found indeed cells with a neuronal identity migrating from the diencephalon into the midbrain of the developing chicken embryo. This suggests that the mesDA system of higher vertebrates descends at least in part from the ancestral DA neurons found in the anamniote diencephalon.

2.2 Molecular markers for mesDA neuron development in vertebrates

In spite of the phylogenetic differences in the origin of DA neurons between anamniotes and amniotes, the question remains whether the molecular mechanisms controlling their generation are conserved among vertebrates. In particular, the knowledge of the molecular and/or genetic cascades necessary for the development of mesDA neurons in different species reveals evolutionary conserved and therefore essential pathways.

The best studied amniotic vertebrate with respect to the development of mesDA neurons is the mouse. The first detectable marker for proliferating mesDA precursors in the embryonic day (E) 9.5 mouse embryo is the retinoic acid synthesizing enzyme Aldh1a1 (Wallen et al., 1999). Aldh1a1 continues to be expressed in postmitotic mesDA neurons of the embryonic and adult mouse brain (Niederreither et al., 2002; Wallen et al., 1999; Fig. 4). At E10.5, expression of the orphan nuclear receptor Nr4a2 is initiated in a broad area of the embryonic mouse brain, including the ventral midbrain (Wallen et al., 1999; Zetterstrom et al., 1997; Fig. 4). In the absence of *Nr4a2*, transcription of the murine

tyrosine hydroxylase (*Th*) gene is not initiated in postmitotic mesDA precursors (Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Zetterstrom et al., 1997), indicating a strict requirement of this nuclear receptor for *Th* expression. Since Th is the first and rate-limiting enzyme for DA synthesis, its expression identifies a neuron as capable of producing dopamine. The first *Th*-positive neurons appear at E11.5 in the ventral midbrain of the mouse embryo (Wallen et al., 1999; Fig.4). The paired-like homeobox gene *Pitx3* has lately received substantial attention as one of the key factors for mesDA neuron development, especially those of the SN. *Pitx3* is exclusively expressed in postmitotic mesDA neurons from E11.5 onwards (Smidt et al., 1997; Fig. 4), and loss-of-function of this gene in mice revealed its requirement for the maintenance and survival of the SN mesDA neurons (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004a; van den Munckhof et al., 2003). More recent evidence, however, suggests that Pitx3 may be specifically required in the developing SN DA neurons for initiation of *Th* transcription, and the failure to do so leads to their premature death (Maxwell et al., 2005). This is of particular interest as *Pitx3* expression in this region of the developing mouse embryo appears to precede the start of *Th* expression by approx. half a day (our own unpublished observations and Maxwell et al. (2005)). Three other transcription factors of the homeobox

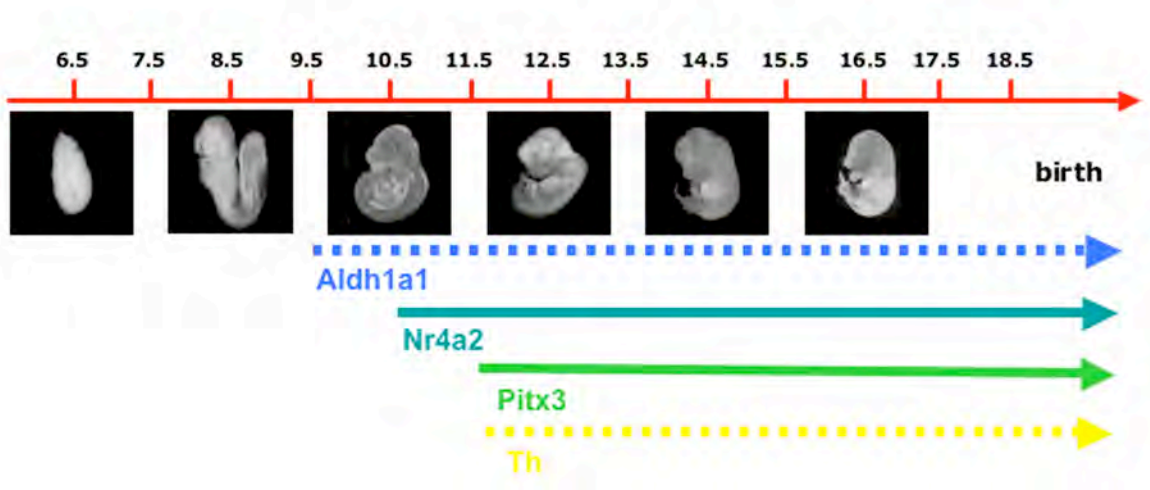

Figure 4: Timescale of the expression onset of some mesDA marker genes in the developing mouse embryo. The first detectable marker for proliferating mesDA progenitors is *Aldh1a1* at E9.5. One day later, transcription of the nuclear receptor *Nr4a2* starts in postmitotic mesDA precursors, followed by expression of *Pitx3* and *Th* at E11.5. Solid bars indicate that the corresponding molecule is necessary for the normal development of mesDA neurons during that time interval. Dotted bars indicate that a direct requirement of the corresponding protein for the development of mesDA neurons has not yet been demonstrated or is not necessary.

family, Lmx1b and Engrailed (En) proteins 1 and 2, are also implicated in mouse mesDA neuron development (Simon et al., 2001; Smidt et al., 2000). Although expression of all three factors is not confined to the ventral midbrain during mouse embryo development, they are later restricted to the SN and VTA in the adult rodent brain (Asbreuk et al., 2002; Simon et al., 2001). All three factors are required for the maintenance and survival but not for the initial induction and differentiation of mesDA neurons in the mouse (Alberi et al., 2004; Simon et al., 2001; Smidt et al., 2000).

Recently, a study investigated the expression and function of the transcription factors Pitx3 and Lmx1b as well as of the nuclear receptor Nr4a2 in the development of the ventral diencephalic DA neurons in the zebrafish (Filippi et al., 2007). Because of the genome duplication in the ancestor of teleosts, zebrafish possess two paralogues for the mouse and chicken genes. Both zebrafish *nr4a2* paralogues, *nr4a2a* and *nr4a2b*, the two *lmx1b* genes (*lmx1b.1* and *lmx1b.2*) and *pitx3* are not expressed in the ventral diencephalic DA neurons, but in a diencephalic territory that may contain precursor cells of this neuronal population (Filippi et al., 2007). The knock-down of both *lmx1b* paralogues led to a specific reduction of neurons in the ventral diencephalic DA cluster. Furthermore, the *lmx1b.1/lmx1b.2* knock-down affected the expression of *pitx3* and *nr4a2* in the ventral diencephalon. However, the knock-down of neither *pitx3* nor the two *nr4a2* genes did impair the development of the ventral diencephalic DA neurons (Filippi et al., 2007). These results indicate that Lmx1b function is required to maintain normal expression of *pitx3* and *nr4a2* in the ventral diencephalon and thereby to establish a competence domain for the development of ascending DA neurons in the zebrafish.

Although the orthologues for *Pitx3* and *Lmx1b* have already been identified in amphibians (*Xenopus laevis*) (Pommereit et al., 2001; Khosrowshahian et al., 2005; Haldin et al., 2003), no expression or functional analysis regarding the development of the DA system was done yet in these species. For the reptiles no data at all is available for any of the orthologues of the mouse mesDA marker genes. The situation found in the chicken (*Gallus gallus*) is approximately the same. The chicken *Lmx1b* gene was first described by Vogel et al. (1995). However, the function of Lmx1b in the development of mesDA neurons has not been addressed yet in the chicken embryo. Although sequence data for the chicken embryo are available from the NCBI and ENSEMBLE databases, no expression or functional analysis was done until today for the marker genes *Pitx3* and *Nr4a2*. However,

in a recent study Lmx1b and Nr4a2 were used as markers for mesDA cells in the chicken embryo (Andersson et al., 2006b).

In the present study, I did a detailed expression analysis of the chicken orthologues for the mouse mesDA marker genes. I found that the initial expression of the mesDA marker genes *Lmx1b, Pitx3* and *Nr4a2* is confined to a diencephalic territory in the chicken embryo. This would be consistent with the hypothesis that the mesDA neurons have a phylogenetic/evolutionary origin in diencephalic progenitors. In addition, the expression profile of *Pitx3* in the chicken embryo has more similarity with the *pitx3* expression of the zebrafish at early stages, whereas later in development chicken *Pitx3* expression is also found in comparable domains to the mouse embryo.

2.3 Molecular mechanisms regulating the development of mesDA neurons in anamniotic and amniotic vertebrates

In the mouse embryo, the mesDA progenitors arise in the midbrain at around embryonic day (E) 9.5 in close vicinity to two signaling centers: the ventral midline or floor plate (FP) and the mid-/hindbrain boundary (MHB) or isthmic organizer (IsO). The FP comprises cells that secrete the glycoprotein sonic hedgehog (Shh). Shh was identified as one important molecule specifying the different neuronal subpopulations along the dorso-ventral axis of the neural tube (Jessell, 2000). Analysis of transgenic mice ectopically expressing the bioactive N-terminal part of the Shh protein or one of the intracellular mediators of the Shh signal, the zinc finger transcription factor Gli1, across the MHB showed that in both mouse mutants, mesDA neurons were ectopically induced in the dorsal midbrain (Hynes et al., 1997). In accordance with this result, an ectopic induction of DA neurons was detectable after the treatment of rat explant cultures with Shh protein (Hynes et al., 1995; Ye et al., 1998). These findings supported the idea that Shh or one of its downstream mediators is sufficient to induce the mesDA neuronal fate in the dorsal midbrain. However, mesDA neurons were not induced in the dorsal hindbrain in these mouse mutants, indicating that additional factors along the A/P axis are required for the specification of mesDA progenitors. In addition, mesDA neurons are missing in *Shh* null mutants and are strongly reduced in *En1-Gli2* conditional knock-outs (Blaess et al., 2006) indicating a requirement of Shh for the normal development of mesDA neurons.

The MHB has been identified as an important secondary organizer that controls patterning events in the developing mid- and hindbrain (Wurst and Bally-Cuif, 2001; Liu and Joyner, 2001). Several secreted factors expressed in the MHB region have been implicated in mesDA neurogenesis, such as the fibroblast growth factor 8 (Fgf8), the transforming growth factors (Tgfs) -α and-ß, and the secreted glycoprotein Wnt1 (Blum, 1998; Farkas et al., 2003; Ye et al., 1998; Castelo-Branco et al., 2003; Prakash et al, 2006). The role of Wnt1 in mesDA neuron development *in vivo* has been addressed in a recent study by the analysis of several transgenic mouse lines that display different mesDA neuron phenotypes (Prakash et al., 2006). Expression of *Wnt1* is found in a ring encircling the neural tube at the isthmic constriction, in the roof plate (RP) of the midbrain and posterior diencephalon, and in the ventral cephalic flexure where mesDA neurons arise (Parr et al., 1993; Panhuysen et al., 2004). Wnt1 has been shown to exert two different functions in mesDA neuron generation. First, Wnt1 is involved in the establishment of the mesDA progenitor domain by maintaining Otx2 expression in the ventral midbrain, which in turn is required for the repression of Nkx2.2 in the FP and basal plate (BP) (Fig. 4A). mesDA neurons are generated within this Nkx2.2 negative territory under the influence of Shh emanating from the FP (Prakash et al., 2006). Second, Wnt1 has a later specifying activity required for the proper differentiation of mesDA precursors into mature mesDA neurons. In the absence of *Wnt1*, only a few Th-positive cells were generated, which did not express the transcription factor *Pitx3* and the dopamine transporter (*Slc6a3*) (Prakash et al., 2006).

Recently, the homeodomain transcription factor Lmx1a was shown to be sufficient and required for the generation of mesDA neurons in the chicken embryo (Andersson et al., 2006b; Ono et al., 2007). The induction of the ectopic mesDA neurons appeared to be Shh dependent, as they were only generated in the ventral part of the chicken midbrain after the overexpression of Lmx1a or in the presence of Shh protein in mouse embryonic stem (ES) cells (Andersson et al., 2006b). Lmx1a seems to mediate its effects in part through the activation of another homeobox gene, Msx1 (Andersson et al., 2006b). Although ectopic expression of Msx1 alone was not sufficient to induce mesDA neurons in the chicken midbrain, it appears to be required for the repression of the most ventral domain of the homeobox gene Nkx6.1, thereby establishing a domain competent for generating mesDA neurons (Andersson et al., 2006b; Fig. 5A). Additionaly, Msx1 is able to prematurely turn

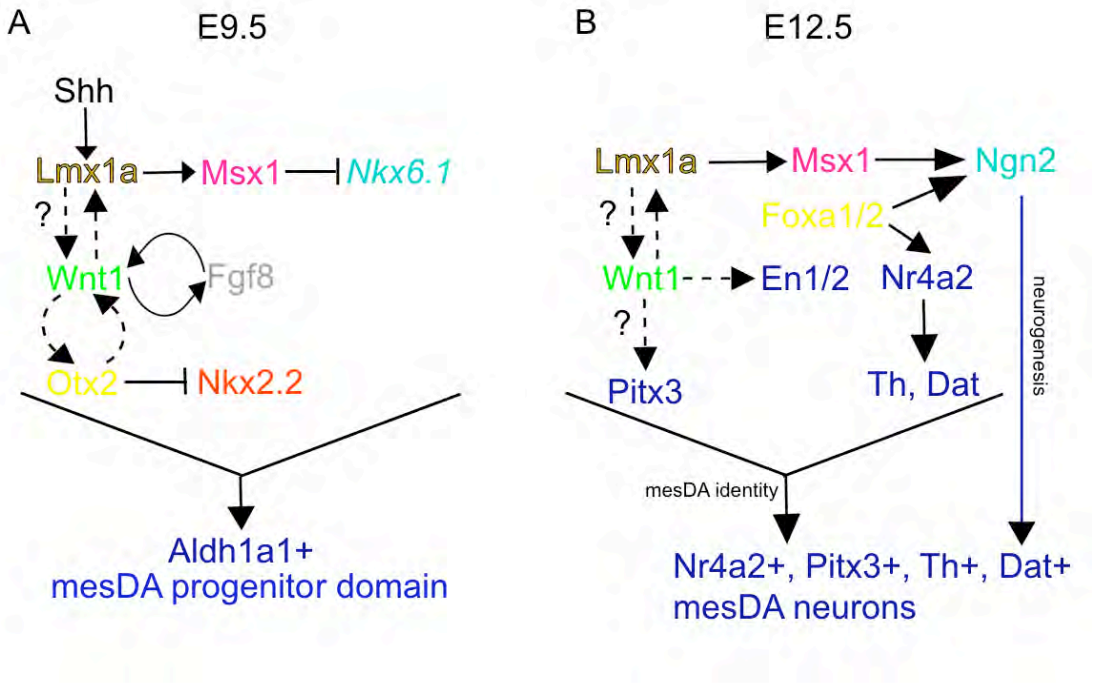

Figure 5: Genetic networks controlling the generation of mesDA neurons in the mouse. (A) At E9.5, Wnt1 (green) together with Shh (black) and Lmx1a (brown) are expressed in the FP and/or adjacent BP of the midbrain. These factors positively control a genetic cascade including *Otx2* (yellow), required for the repression of *Nkx2.2* (red), and *Msx1* (pink), required for the repression of *Nkx6.1* (turquoise). Induction of *Lmx1a* appears to require Shh. The possible interactions between Wnt1 and Lmx1a are not yet clarified. Wnt1 is also required for the maintenance of *Fgf8* (gray) expression and vice versa. (B) At E12.5, Lmx1a positively controls a genetic cascade including *Msx1*, required for the expression of *Ngn2*. Foxa1/2 positively regulates *Ngn2* expression in mesDA progenitors and is required for *Nr4a2* expression in immature mesDA neurons. Wnt1 may regulate/maintain the expression of Pitx3 and En1/2 at this stage. A complementary pathway controlled by Nurr1 (Nr4a2) induces the expression of Th and Dat. The possible interactions between Lmx1a and Wnt1 are yet unknown. Modified from Prakash and Wurst (2006).

on the expression of the proneural factor Ngn2 and thus mesDA neurogenesis when expressed at stages earlier in mice carrying a *Shh enhancer-Msx1* transgene. Ngn2 has been shown to be a key regulator for mesDA neurogenesis (Andersson et al., 2006b; Kele et al., 2006; Fig 5B). In the absence of *Ngn2*, the mesDA neuronal population was initially reduced to less than 20%, but recovered postnatally probably due to a partial compensation by Mash1, another proneural factor (Andersson et al., 2006b; Kele et al., 2006). In a recent study, Ferri et al. (2007) showed that the expression of Ngn2 is regulated by the forkhead/winged helix transcription factors Foxa1 and Foxa2 (Fig. 5B). Foxa2 has been

shown to be one of the targets of Hh signaling (Sasaki et al., 1997). Expression of *Foxa2* is absent from the ventral CNS of mouse mutants lacking activity of the Hh transcriptional effector Gli2, and exogenous Shh can induce ectopic *Foxa2* expression (reviewed by Strähle et al., 2004). Reciprocally, ectopic Foxa2 activity can induce *Shh* expression (Hynes et al., 1995). In conclusion, a complex genetic network including the Hh- and Wnt-signaling pathways is responsible for the generation of mesDA neurons in the mouse embryo. Whether there exists an interaction between these two pathways is not yet clarified.

In the zebrafish, DA neurons corresponding to the mesencephalic groups are located in the basal diencephalon (Kaslin and Panula, 2001; Rink and Wullimann, 2001, 2002a, b). An analysis of several signaling pathway mutants uncovered genetic components involved in the development of DA neurons in the zebrafish. Thus, Holzschuh and colleagues (2003) showed that the ventral diencephalic DA neurons require transforming growth factor type ß (Tgf-ß)/Nodal signaling. A strict requirement was not confirmed for the fibroblast growth factor (Fgf)- and the Hedgehog (Hh)- pathways (Holzschuh et al., 2003).

Classical anatomical and connectivity studies were done in amphibians (Marin et al., 1998) and reptiles (reviewed in Median and Reiner, 1995). Investigations of the signaling pathways responsible for the proper development of mesDA neurons are still missing for these species. Interestingly, the salamander brain possesses an astonishing regenerative capacity after injury (Parish et al., 2007). It is likely that the regeneration of mesDA neurons in salamander obeys the same cues required for the normal development of mesDA neurons in this species. However, these signals still await identification.

An old model organism in developmental biology is the chicken embryo. Histochemical, pathway tracing and neuropeptide/neurotransmitter localization studies in birds were already done during the 1970^{s} and 1980^{s} (reviewed in Smeets and Gonzalez, 2000). The accessibility of the chicken embryo in the egg allows the easy study of the early development of mesDA neurons. This fact makes it even more astonishing that not much was known about the molecular mechanisms controlling the generation of mesDA neurons in the chicken embryo. Only Farkas et al. (2003) showed an involvement of Tgf-ß- and Shh- signaling in the induction and maintenance of mesDA neurons in the chicken embryo. The ability of Shh to induce ectopic DA neurons in the dorsal midbrain of the chicken embryo was also described by Watanabe and Nakamura (2000). Thus, Tgf-ß- and Hh-

signaling are necessary for the development of mesDA neurons in the chicken embryo. However, data about other pathways and the precise genetic cascades are still missing.

I identified new pathways and molecular interactions required for mesDA neuron development in the chicken embryo in the present work. By using gain- and loss-of-function experiments I could show that the Wnt-pathway is another key component in early mesDA neuron development, and that the Shh-signaling pathway required for mesDA neuron development (Farkas et al., 2003) may be downstream and/or interacting with the Wnt-pathway in the chicken embryo.

2.4 Aims of the present work

1) Precise spatio-temporal analysis of mesDA neuron development in the early chicken embryo using the chicken orthologues of the different mouse mesDA marker genes.
2) Expression analysis of selected Wnt genes and functional analysis of the Wnt- and Hh- pathways in mesDA neuron development in the early chicken embryo.

3 Results

3.1 Development of the mesDA neurons in the chicken embryo

For the mouse embryo, several factors have been identified to play a role in the specification and differentiation of the mesDA neurons, which are now used as markers for different steps of mesDA development. However, until today no data was available for the chicken orthologues of the mesDA marker genes *Lmx1b, Aldh1a1, Nr4a2* and *Pitx3* during chicken embryonic development. To investigate the early development of mesDA neurons in the chicken embryo, a prerequisite is a precise analysis of the temporal and spatial expression patterns for the mesDA marker genes and the confirmation that they can also be used as mesDA markers in the chicken embryo. Therefore, the corresponding chicken orthologues were cloned and analysed for their expression patterns during early neural development.

3.1.1 Expression of the chicken orthologues for mouse mesDA marker genes during early chicken neural development

3.1.1.1 Expression of the chicken orthologues for mouse mesDA marker genes at embryonic day 3.5 of chicken development

The analysis was started at embryonic day (E) 3.5 of chicken development, which corresponds to mouse E11.5, the stage when the expression of all mesDA marker genes mentioned above are detectable in the ventral midbrain of the mouse neural tube (reviewed in Prakash and Wurst, 2006). *In situ* hybridisation (ISH) of consecutive sagittal and coronal sections with radioactively labeled probes for the different chicken orthologues of the mesDA marker genes revealed that *Pitx3, Nr4a2* and *Lmx1b* mRNAs were detected in the E3.5 chicken embryo (Fig. 6). *Pitx3* was strongly expressed throughout the neuroepithelium in the ventral midline of the rostral part of the cephalic flexure (Fig. 6B, B´). Furthermore, *Nr4a2* expression was detected at this stage of chicken embryonic development in the same region of the rostral cephalic flexure where *Pitx3* was transcribed

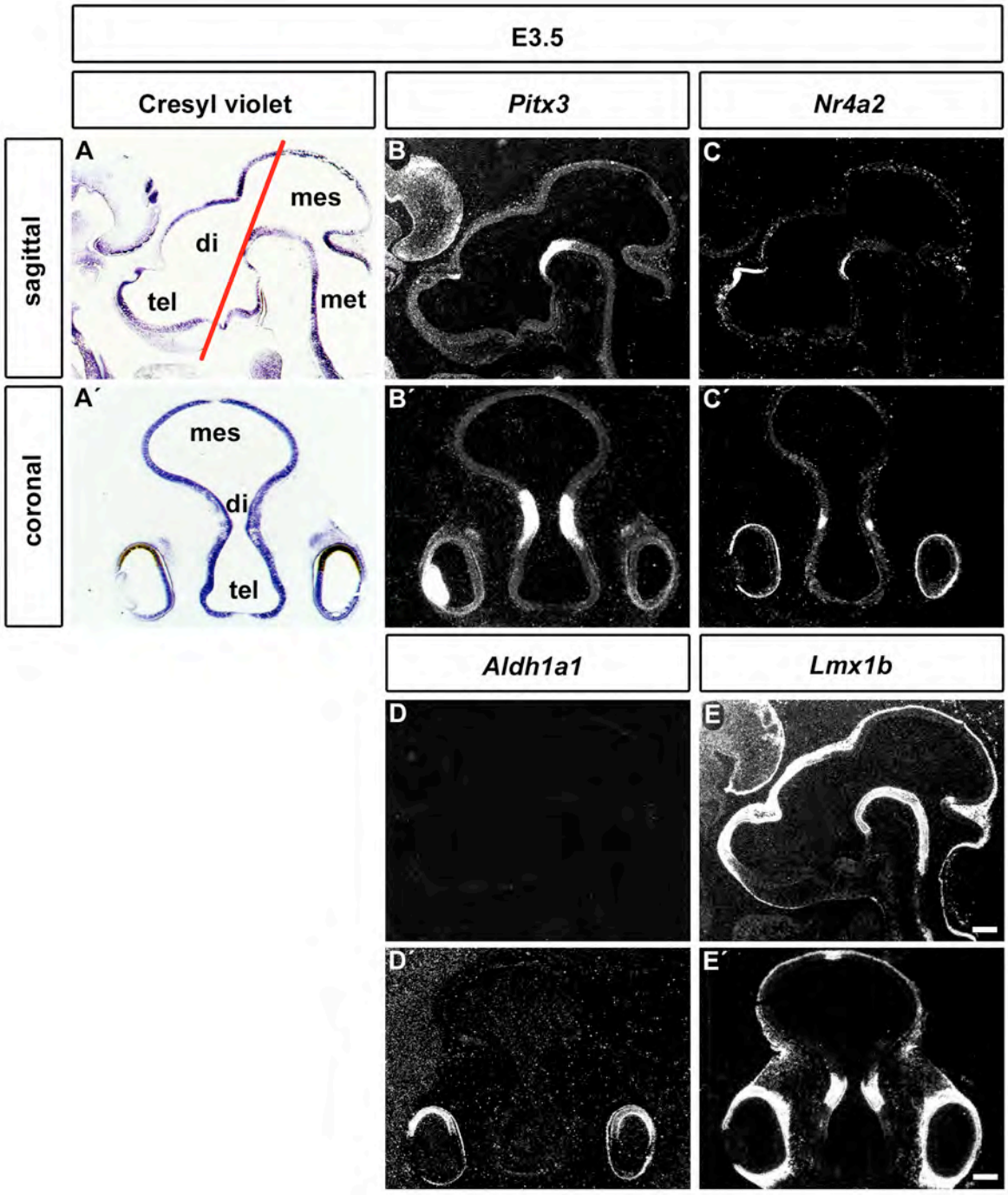

Figure 6 : Expression of chicken orthologues for mouse mesDA marker gene expression in the E3.5 chicken embryo. (A-E) Consecutive midsagittal sections of E3.5 chicken embryos, anterior is to the left. (A) Brightfield image of (B). The red line in (A) depicts the level of coronal sections in (A´-E´). Darkfield images of sections hybridised with probes for *Pitx3* (B), *Nr4a2* (C), *Aldh1a1* (D) and *Lmx1b* (E). (A´-E´) Consecutive coronal sections of E3.5 chicken embryos, dorsal is at the top. (A´) Brightfield image of (D´). The sections were hybridised with *Pitx3* (B´), *Nr4a2* (C´), *Aldh1a1* (D´) and *Lmx1b* (E´). Abbreviations: di, diencephalon; mes, mesencephalon; met, metencephalon; tel, telencephalon. Scale bars: 200 μm.

(Fig. 6C, C´). However, the *Nr4a2*-positive domain was much smaller than the *Pitx3*-expressing territory and appeared to be confined to the mantle zone of the neuroepithelium (Fig. 6B´, C´). Expression of *Nr4a2* was also detected in the dorsal telencephalon at this stage (Fig. 6C). In contrast to the more restricted expression domains of *Pitx3* and *Nr4a2* in the ventral neural tube of the E3.5 chicken embryo, *Lmx1b* was strongly expressed in the floor plate of the chicken fore-, mid- and hindbrain, in the roof plate of the fore- , mid- and hindbrain and in a ring encircling the isthmus (Fig. 6E, E´; Adams et al., 2000). Expression of the *Aldh1a1* gene was not detected at all in the developing neural tube of the E3.5 chicken embryo, although a clear signal was seen in the dorsal neural retina of the developing eye (Fig. 6D, D´), in line with previous reports (Blentic et al., 2003; Suzuki et al., 2000).

To rule out the possibility that one of the other two chicken *Aldh1a* family members, *Aldh1a2* (Tsukui et al., 1999) and *Aldh1a3* (Suzuki et al., 2000), may be expressed instead of *Aldh1a1* in the ventral fore- or midbrain, and that this domain may have been missed in previous studies by whole mount *in situ* hybridisation (ISH) and immunohistochemical procedures (Berggren et al., 1999; Blentic et al., 2003; Suzuki et al., 2000; Swindell et al., 1999), the decision was made to reinvestigate the expression domains by means of radioactive ISH and focus on the ventral mesencephalic territory in the chicken embryo.

However, expression of neither *Aldh1a1* paralogues was detected in the embryonic chicken neural tube at E3.5 (Fig. 7), although *Aldh1a2* was expressed in the surrounding

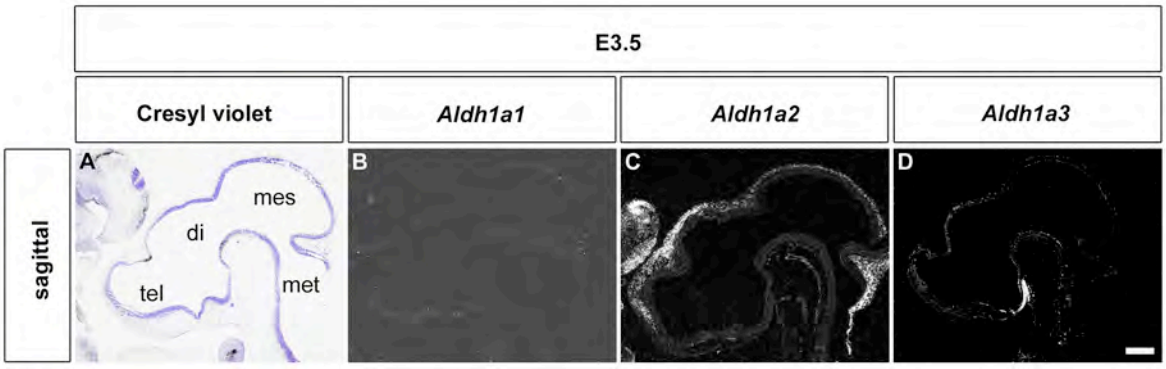

Figure 7: Chicken *Aldh1a* family members are not expressed in the chicken neural tube. (A-D) Midsagittal sections of E3.5 chicken embryos. (A) Brightfield image of (B). The darkfield images were taken from consecutive sections hybridised with probes for *Aldh1a1* (B), *Aldh1a2* (C) and *Aldh1a3* (D). Abbreviations: di, diencephalon; mes, mesencephalon; met, metencephalon; tel, telencephalon. Scale bar: 200 μm.

head mesenchyme (Fig. 7C) and *Aldh1a3* was strongly expressed in Rathke's pouch (Fig. 7D), as partly reported before (Blentic et al., 2003; Suzuki et al., 2000).

3.1.1.2 Expression of the chicken orthologues for mouse mesDA marker genes at E5 of embryonic development

The chicken brain has developed into a more elaborate architecture at E5, which complicates the precise spatial mapping of the expression patterns. As chicken *Fgf8* delimits the caudal border of the MHB (Shamim et al., 1999), it was used for assessing the expression of *Lmx1b*, *Nr4a2* and *Pitx3* relative to the MHB. At E5, the expression of *Pitx3* and *Nr4a2* was detected in the entire cephalic flexure of the chicken embryo, with a sharp caudal border that corresponded to the position of the MHB abutting the *Fgf8* expression domain (Fig. 8B, D, E). Notably, the most anterior part of the cephalic flexure showed the highest expression of *Pitx3* (Fig. 8B, D). In contrast, transcription of *Nr4a2* was equally strong throughout the entire ventral di- and mesencephalon at this stage (Fig. 8E). The expression of these two chicken orthologues appeared to be restricted to the outer margin of the neuroepithelium corresponding to the marginal zone of the ventral neural tube (Fig. 8B, D, E), suggesting that they were expressed only by the postmitotic progeny that had migrated out. The *Lmx1b* mRNA was expressed in the entire ventral mid- and hindbrain at E5, but had a stronger expression in the cephalic flexure than in the ventral hindbrain (Fig. 8F). Since *Th* expression was not detected in the cephalic flexure at E5 (data not shown), the *Lmx1b+/Nr4a2+/Pitx3+* cells were considered to be DA precursors, that had not differentiated into mature DA-synthesizing neurons.

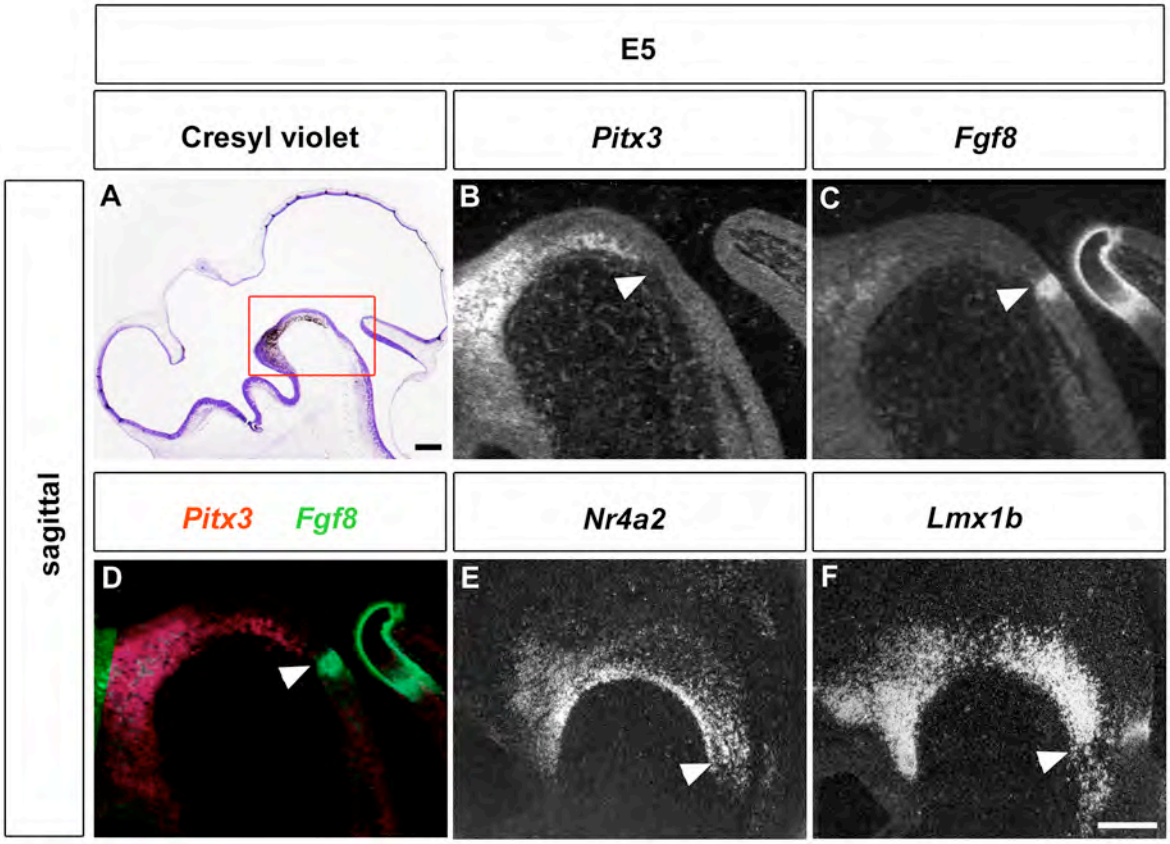

Figure 8: Expression of chicken orthologues for mouse DA marker genes in the E5 cicken embryo. (A-F) Midsagittal sections of E5 chicken embryos. (B-F) Close-up views of the cephalic flexure (red box in (A)). The arrowheads show the position of the ventral MHB. (A) Brighfield image of (A). Sections were hybridised with *Pitx3* (B), *Fgf8* (C), *Nr4a2* (E) and *Lmx1b* (F). (D) Pseudocoloured overlay of the corresponding darkfield images in (B, C) hybridised with probes for *Pitx3* (red) and *Fgf8* (green). Scale bars: 200 µm.

It should be noted that the transcription of *Pitx3* was not restricted to the ventral mes- and diencephalon at this stage. *Pitx3* showed a very prominent expression domain in the dorsal forebrain of the E5 chicken embryo, that appeared to be a dorsal expansion in the zona limitans intrathalamica (ZLI). To confirm whether this is indeed the case, a double whole mount ISH was performed on E5 chicken brains with probes for *Pitx3* (purple) and *Shh* (red). Shh has a prominent dorsal extension in the ZLI of the diencephalon (Echevarria et al., 2003). A complete overlap of the two expression domains was found in the ZLI (Fig. 9A-C).

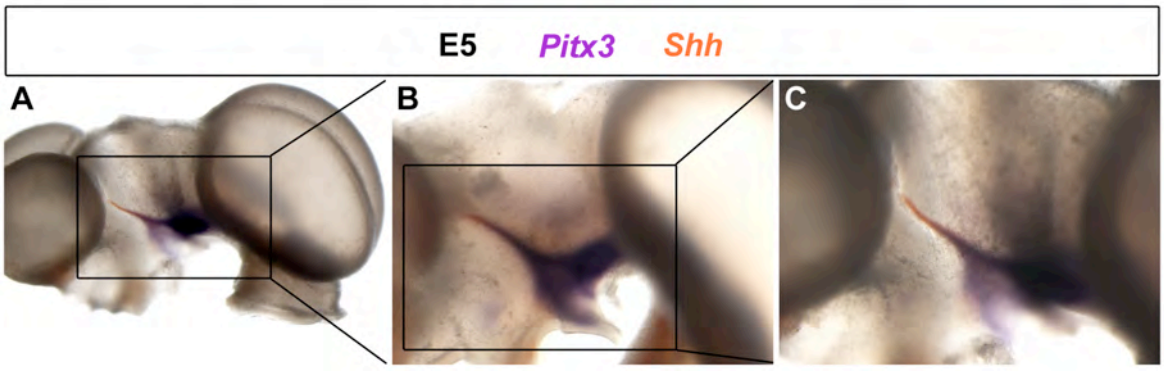

Figure 9: *Pitx3* is expressed in the ZLI. (A-C) Whole mount ISH on E5 chicken brain with probes for *Pitx3* (purple) and *Shh* (orange). (B) is a close-up of the boxed area in (A), (C) of the boxed area in (B). A clear overlap of the two expression domains of *Pitx3* and *Shh* was detected in the ZLI and in the cephalic flexure.

3.1.1.3 Expression of chicken orthologues of mouse mesDA marker genes at E6.5 of embryonic development

Expression of the *Th* gene was detected in the ventral midbrain of the chicken embryo at E6.5 (equivalent to mouse E13.5) for the first time (Fig. 10B, B´). At this stage, expression of *Th* was confined to the caudal part of cephalic flexure/ventral midbrain close to the MHB, as assessed by detection of *Fgf8* on consecutive sagittal sections (Fig. 10C, inset). Hybridization with probes for *Pitx3* and *Nr4a2* showed that both transcripts were now confined to the posterior ventral midbrain with a sharp caudal border at the level of the MHB (Fig. 10C, D). The expression domain of both genes, however, was still broader than the *Th* domain within the same region and extended into the more rostral part of the ventral midbrain (*Pitx3*) or even into the diencephalon (*Nr4a2*) (Fig. 10C, D). Consecutive coronal sections through the caudal midbrain of E6.5 chicken embryos revealed that the *Th*-expressing cells were located in two lateral domains within the marginal zone and were sparing the ventral midline of the neural tube (Fig. 10B´), whereas both *Pitx3* and *Nr4a2* were expressed in a much broader domain extending from the ventral midline (floor plate) to more lateral regions of the ventral neural tube (Fig. 10C´, D´). Both the *En1* and *Lmx1b* genes appeared to be expressed in overlapping domains of the ventral midbrain together with the other chicken orthologues of mouse DA marker genes, as assessed on consecutive sagittal and coronal sections (Fig. 10A, A´, E, E´). Nevertheless, the expression of *En1* extended into the rostral hindbrain and that of *Lmx1b* continued into the ventral

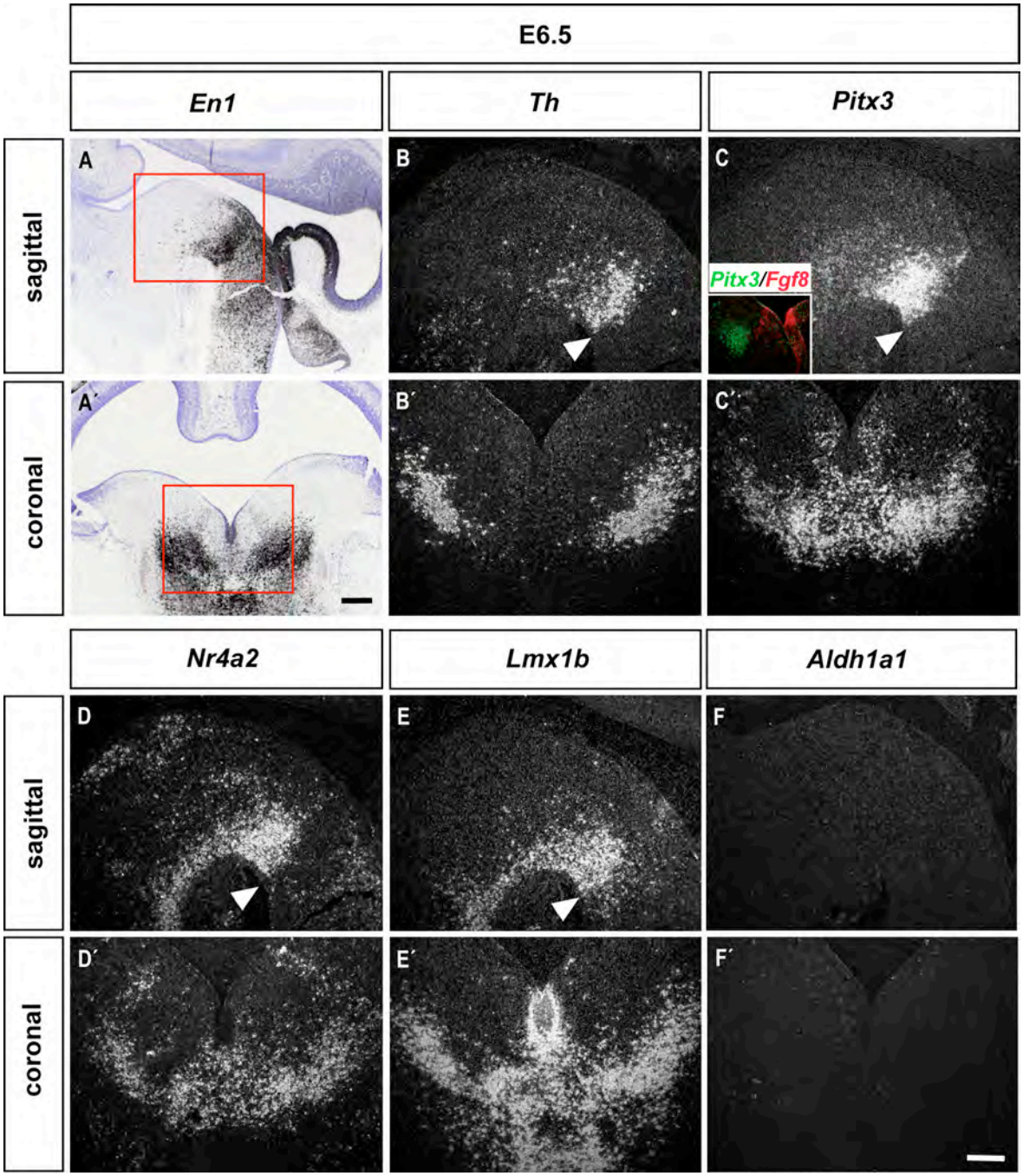

Figure 10: Expression of the chicken orthologues of mouse mesDA marker genes at E6.5. (A-F) Consecutive midsagittal sections of E6.5 chicken embryo brains. The arrowhead points at the MHB, as determined by *Fgf8* (green) expression (inset in C). (A) Brightfield image of a section hybridised with *En1*. The red rectangle depicts the region of the darkfield images shown in (B-F). Sections were hybridised with probes for *Th* (B), *Pitx3* (C), *Nr4a2* (D), *Lmx1b* (E) and *Aldh1a1* (F). (A´-F´) Consecutive coronal sections of E6.5 chicken embryo brains. (A´) Brightfield image of a coronal section hybridised with *En1,* the red rectangle depicting the region of the darkfield images shown in (B´-F´). The consecutive sections were hybridised with *Th* (B´), *Pitx3* (C´), *Nr4a2* (D´), *Lmx1b* (E´) and *Aldh1a1* (F´). No expression of *Aldh1a1* was detected in the ventral midbrain of the chicken embryo at this stage. Scale bars: 200 μm.

diencephalon (Fig. 10A, E). Notably, transcription of the chicken orthologues except *Th* analyzed at this stage of embryonic development was not restricted to the outer rim (marginal zone) of the neural tube, but they also showed expression in the ventricular zone of the medial floor plate (Fig. 10C′, D′, E′). Since the cells expressing all chicken orthologues (*Lmx1b*, *Nr4a2*, *Pitx3*, *Th*) were located in the caudo-ventral midbrain of the chicken embryo in a region where the Th- and DA-expressing chicken mesDA neurons have been mapped by Puelles and Medina (1994), we concluded that they are mesDA neurons.

Pseudocoloured overlays of either the *Th*/*Pitx3* or *Th*/*Nr4a2* expression domains in the ventral midbrain on consecutive coronal sections clearly demonstrated that there was only a partial overlap between the lateral *Th*- and *Pitx3*-expressing domains (Fig. 11A). This is also true for the *Th*- and *Nr4a2*-expressing domains. The overlap showed, that there are also *Nr4a2*+ domains that are *Th*– (Fig. 11B). Expression of *Pitx3* and *Nr4a2* at this stage suggested that the postmitotic *Th*+ cells originate at least in part from the *Pitx3*+/*Nr4a2*+ progenitors/precursors located in the ventricular and subventricular zone of the ventral midbrain.

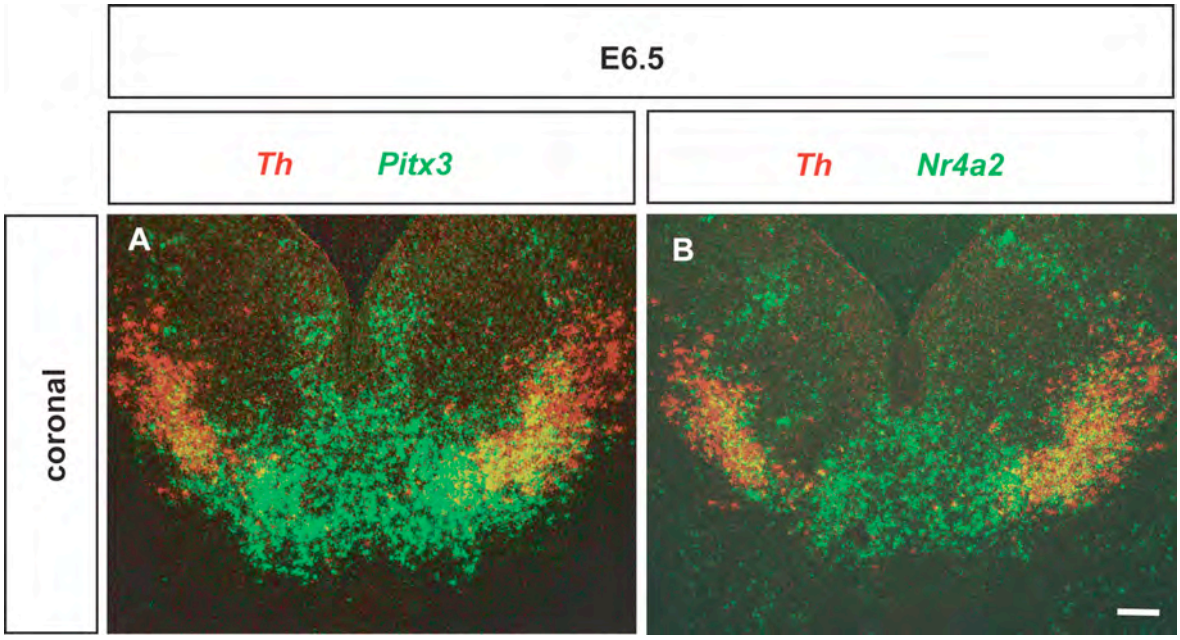

Figure 11: *Pitx3* and *Nr4a2* are expressed in a broader pattern than *Th* in E6.5 chicken embryos. Pseudocoloured overlays of the corresponding darkfield images (Fig. 6B′, C′, D′) from coronal sections hybridised with probes for (A) *Pitx3* (green) and *Th* (red), or (B) *Nr4a2* (green) and *Th* (red). Overlapping domains appear in yellow. Scale bar: 200 µm.

Taken together, the expression analysis of the chicken orthologues of mouse mesDA marker genes revealed spatial and temporal differences of the expression pattern between the two species. No expression at all was found for *Aldh1a1* in the embryonic neural tube, whereas *Pitx3* and *Nr4a2* were initially expressed in a very rostral domain of the ventral cephalic flexure of the early chicken embryo. In addition, *Pitx3* was expressed in the ZLI of the chicken embryo. At later stages, *Pitx3* and *Nr4a2* are expressed in broad domains of the ventral midbrain only partially overlapping with the *Th* expression domain.

3.1.2 *Pitx3* is the earliest chicken orthologue for mouse mesDA marker genes expressed in the chicken embryo

To assess precisely at which time-point the expression of the chicken orthologues of the mouse mesDA marker genes starts, an ISH for *Pitx3, Nr4a2, Lmx1b* and *Aldh1a1* was performed at stage E2.5. *Pitx3* was the first gene to be expressed in the E2.5 chicken embryo (Fig. 12B-B´). None of the other chicken orthologs except *Lmx1b* were expressed at this early stage, in line with an earlier patterning function of Lmx1b in the midbrain (Fig. 12D-D´; Adams et al., 2000; Matsunaga et al., 2002). A strong *Lmx1b* expression was detected in the roof and floor plate of the fore-, mid- and hindbrain at E2.5 (Fig. 12D-D´´). At this stage, *Pitx3* was already strongly expressed throughout the neuroepithelium in the rostral part of the cephalic flexure (Fig. 12B, B´). No expression of *Pitx3* was detected in the ventral midbrain at this stage (Fig. 12B´´). Neither *Nr4a2* (Fig. 12C-C´´), nor *Aldh1a1* (Fig. 12E-E´´) were expressed in the neural tube at this early stage.

In summary, these data strongly suggest that *Pitx3* is the earliest marker for mesDA neurons in the chicken embryo. Although *Pitx3* is first expressed in a diencephalic region, its expression shows an overlap at later stages in the ventral midbrain with *Th* (Fig. 11A), the marker gene for fully differentiated DA neurons. In addition, *Pitx3* shows a co-expression with all genes known to play a role in mesDA neuron development in the mouse embryo at early (E2.5: *Lmx1b*) and at later (E3.5 and E5: *Lmx1b, Nr4a2*; E6.5: *Lmx1b, Nr4a2, En1, Th*) stages of embryonic development.

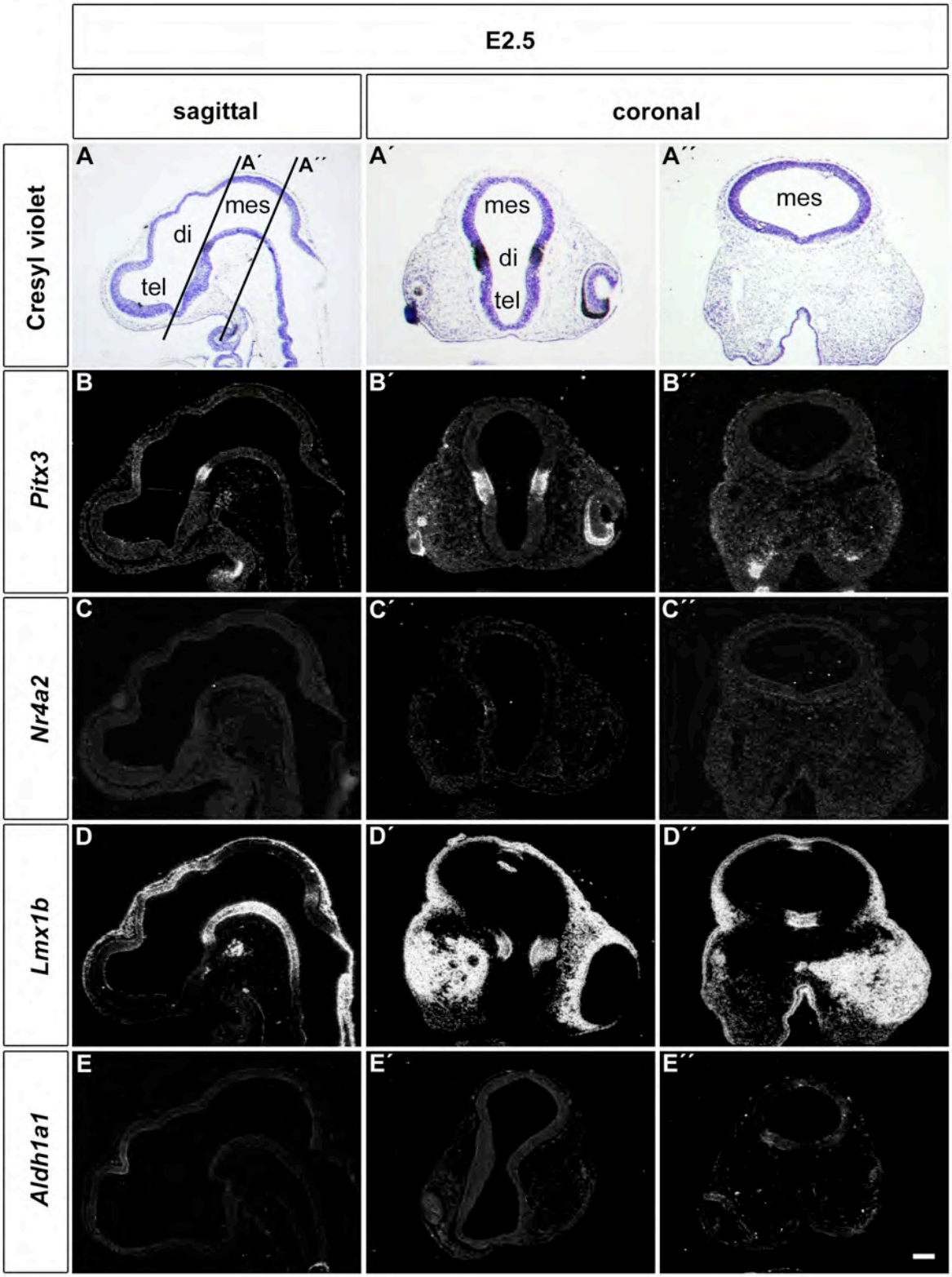

Figure 12: Expression of the chicken orthologues of mouse mesDA marker genes at E2.5. (A-A´) Brightfield images of (B-B´). The black lines indicate the level of sections in (A´-E´) and (A´´-E´´), respectively. (A´´) Brightfield image of (C´´). (A-E) Sagittal sections of E2.5 chicken embryos hybridised with probes for *Pitx3* (B), *Nr4a2* (C), *Lmx1b* (D) and *Aldh1a1* (E). (A´-E´´) Coronal sections of E2.5 chicken embryos hybridised with probes for *Pitx3* (A´,A´´), *Nr4a2* (B´,B´´), *Lmx1b* (C´,C´´) and *Alh1a1* (E´,E´´). Abbreviations: di, diencephalon; mes, mesencephalon; tel, telencephalon. Scale bar: 200 μm.

3.1.3 Chicken *Pitx3* is initially expressed in a ventral diencephalic territory

The very rostral location of the *Pitx3* expression domain in the neural tube of the E2.5 and E3.5 chicken embryo (Fig. 6 and 12) suggested that the *Pitx3*- positive neural precursors were induced in a region corresponding to the diencephalon, which encompasses prosomeres (p) 1-3 according to the prosomeric model established by Puelles and Rubenstein (Puelles and Rubenstein, 2003; Rubenstein et al., 1994). To establish this conclusively, a double ISH was performed for *Pitx3* and two other marker genes, *Pax6* and *Shh*. *Pax6* is expressed throughout the forebrain up to the diencephalic (p1)/mesencephalic (mes) boundary (Matsunaga et al., 2000), and *Shh* is expressed in the ventral midline of the neural tube comprising the floor and basal plate of the midbrain and caudal diencephalon (reviewed by Echevarria et al., 2003). In addition, *Shh* expression has a prominent dorsal extension in the ZLI, which delimits the boundary between p2 and p3 (Echevarria et al., 2003). At E3.5, the expression of *Pitx3* was confined to a region of the ventral neural tube

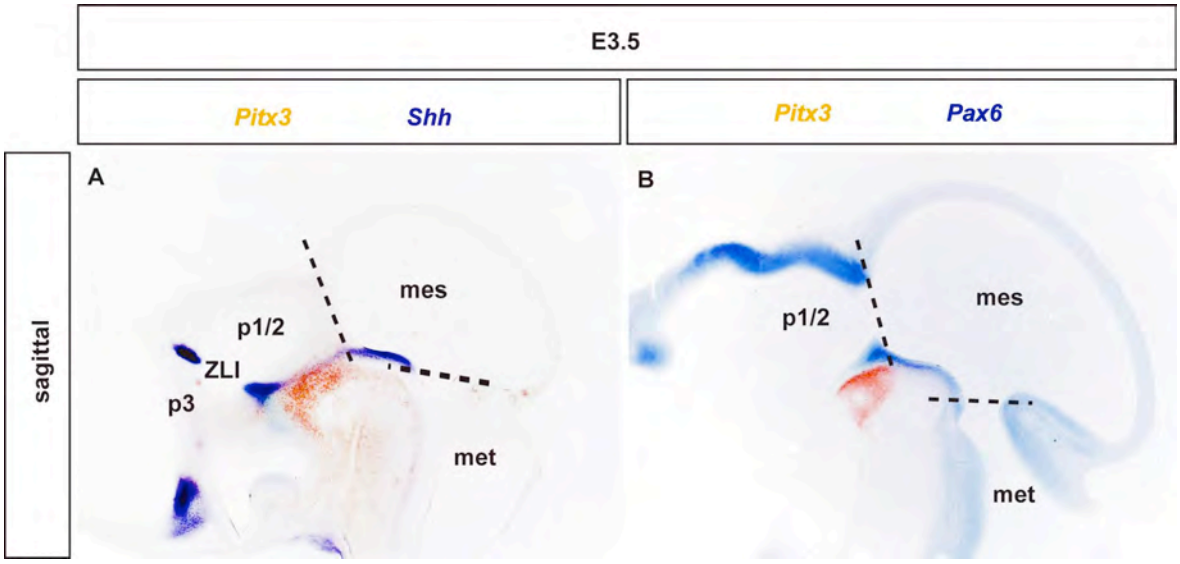

Figure 13: Chicken *Pitx3* is initially expressed in a ventral diencephalic territory. (A, B) Sagittal vibratome section of E3.5 chicken embryos hybridised with riboprobes for *Pitx3* (red in A, B) and *Shh* (dark blue in A) or *Pax6* (blue in B). The red *Pitx3* signal is confined to the p1/2 domain of the diencephalon delimited anteriorly by the *Shh*+ ZLI (A) and posteriorly by the *Pax6*+ p1/mes boundary (B). Abbreviations: mes, mesencephalon; met, metencephalon; p, prosomere; ZLI, zona limitans intrathalamica.

between the *Shh*+ ZLI (Fig. 13A) and the *Pax6*+ p1/mes boundary (Fig. 13B), indicating that *Pitx3* is initially expressed within the p1/2 domain of the diencephalon. *Pitx3* is not expressed in the ventral mesencephalon of the chicken embryo at this stage.

3.1.4 Chicken *Pitx3* is expressed in proliferating cells in the diencephalon

I next asked whether *Pitx3* is expressed in proliferating cells, because of the very early expression which appeared to cover the whole neuroepithelium. Therefore, BrdU short pulse-labeling was combined with an ISH for *Pitx3*. Thereby, only those cells undergoing DNA replication in the S-phase of the cell cycle during the short labeling period incorporated the thymidine-analogue BrdU. Double labeled cells for *Pitx3* (purple) and BrdU (brown) were indeed confirming an expression of *Pitx3* in proliferating cells in the diencephalon (Fig. 14C, arrowheads).

In addition, a double labeling for *Pitx3* (Fig. 15, black) and HuC/D (Fig. 15, red) was performed. The RNA binding proteins HuC/D are expressed in early differentiating neurons (Wakamatsu and Weston, 1997). Therefore, the exclusion of HuC/D from *Pitx3*+ cells in the ventricular zone (VZ) and subventricular zone (SVZ) indicates that *Pitx3* is expressed in undifferentiated and still proliferating cells. Midsagittal sections of the double labeled brains revealed differences in the expression pattern of *Pitx3* along the A/P axis. In the ventral diencephalon, *Pitx3* was expressed broadly throughout the whole neuroepithelium, whereas in the ventral midbrain expression of *Pitx3* was detected only in a ventral stripe (Fig. 15A). A close-up of this mesencephalic area revealed a *Pitx3* expression exclusively in the mantle zone (MZ) (Fig. 15a). Coronal sections at the level of the diencephalon revealed a strong expression of *Pitx3* in the VZ, SVZ and intermediate zone (IZ) of the neuroepithelium (Fig. 15b). In the diencephalon, an overlap of *Pitx3* with the HuC/D expression was detected only in the IZ (Fig. 15b). However, a look at coronal sections at the midbrain level showed no expression of *Pitx3* in the VZ/SVZ at all (Fig. 15c). Interestingly, the co-labeling with HuC/D revealed that *Pitx3* expression was completely overlapping with a strong HuC/D signal in the MZ of the ventral midbrain (Fig. 15c).

The strong expression of *Pitx3* in the VZ/SVZ of the diencephalon but not in the

mesencephalon and at the same time the clear overlap of *Pitx3* with the HuC/D staining in the MZ of the midbrain suggests that cells born in the diencephalon have migrated from the fore- into the midbrain and furthermore, that the postmitotic *Pitx3*+/HuC/D+ cells in the mesencephalon at this stage did not derive from *Pitx3*+ precursors in the mesencephalon.

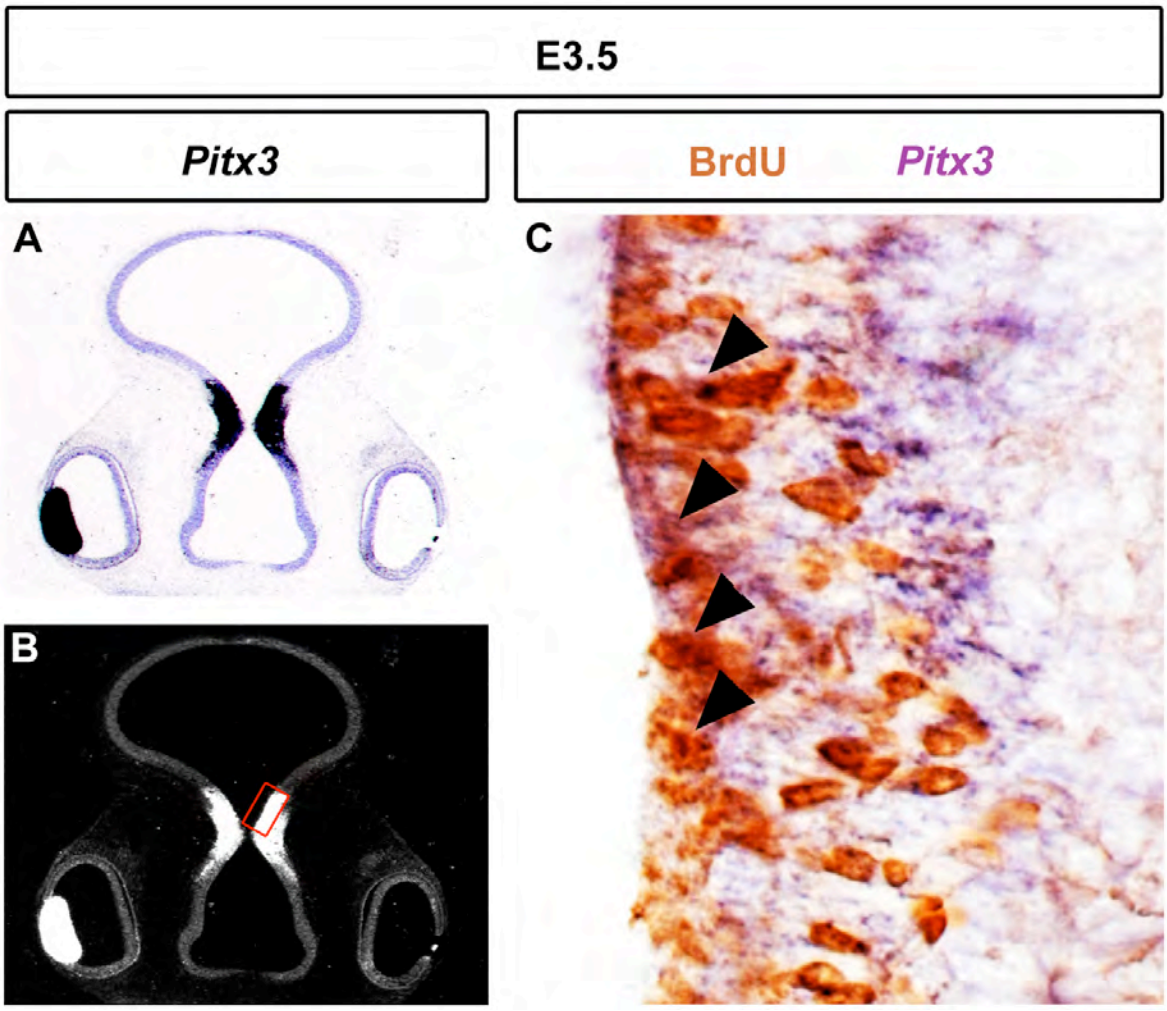

Figure 14: *Pitx3* is expressed in proliferating cells. (A, B) Brightfield (A) and darkfield (B) image of a coronal section of E3.5 chicken embryos hybridised with a probe for *Pitx3*. Red box in (B) shows the corresponding area of a coronal section from a chicken embryo after IHC for BrdU (brown) and WISH for *Pitx3* (purple) shown in (C). Arrowheads indicate double labeled cells.

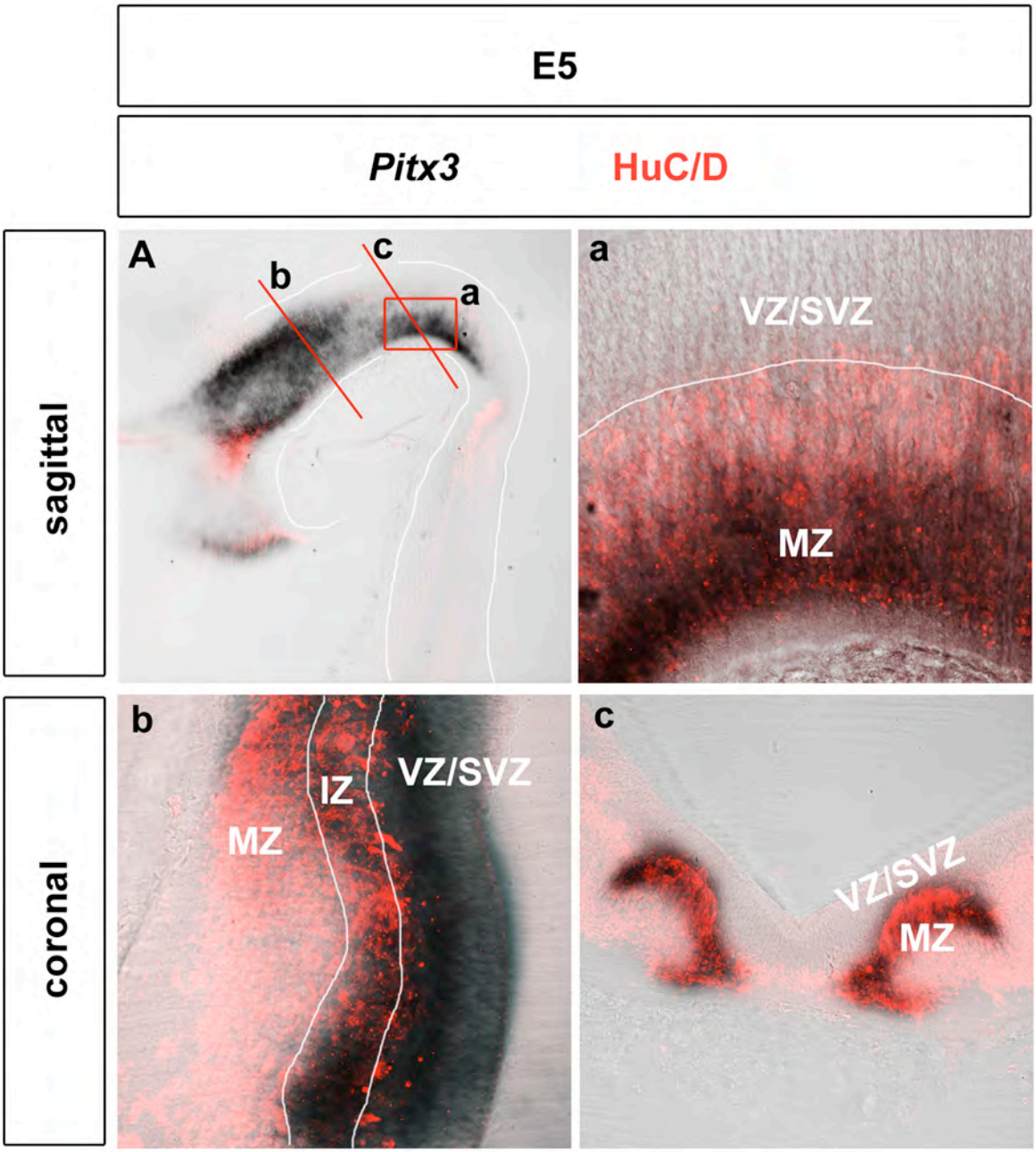

Figure 15: *Pitx3* is expressed in proliferating cells only in the diencephalon. (A) Sagittal section of E5 chicken embryonic brain hybridized with a *Pitx3* ISH probe (black) and superimposed with the fluorescent image after HuC/D immunolabeling (red). The red rectangle in (A) shows the part of the ventral midbrain which is magnified in (a). The red lines in (A) indicate the plane of sections shown in (b) and (c). (a) In the ventral midbrain, *Pitx3* was expressed only in the MZ overlapping with HuC/D expression, whereas the VZ/SVZ was devoid of *Pitx3* and HuC/D. (b) Coronal section of the diencephalon showed an overlap between *Pitx3* and HuC/D only in the IZ. (c) Coronal sections of the mesencephalon showed a complete overlap of *Pitx3* and HuC/D in the MZ. Abbreviations: IZ, intermediate zone; MZ, mantle zone; SVZ, subventricular zone; VZ, ventricular zone.

3.1.5 Do neural precursors migrate from the ventral diencephalon into the midbrain?

The previous results suggested that, besides a *de novo* induction of the corresponding genes, the *Pitx3*+ cells found in the midbrain at later stages could partly also be due to migration of these cells. Therefore, I asked whether a caudal migration of neuronal precursors from the diencephalon into the midbrain takes place during early chicken embryonic development. To answer this question the early *Pitx3*+ diencephalic neural precursors were fate-mapped by electroporating an NLS-eGFP-expressing vector into the *Pitx3*+ diencephalic region to follow the fate of the labeled cells.

The electroporation of the NLS-eGFP construct into the ventral diencephalon was done at E2.5, the time point when *Pitx3* expression is first detected in this area. eGFP- labeled cells migrating caudally towards the midbrain were detected after 1d (Fig. 16A, arrow). The electroporation site in the diencephalon was clearly identified by the eGFP stained neuroepithelium (Fig. 16A, arrowhead). After 4 days of incubation, the chicken brain has grown enormously. However, the electroporation site in the forebrain was still clearly recognizable in the ventral diencephalon (Fig. 16B, arrowhead). Strikingly, eGFP+ cells were also detected in the ventral midbrain (Fig. 16B). A closer look revealed that the eGFP-labeled cells in the ventral midbrain displayed a neuronal morphology with labeled processes (Fig. 16C). To confirm their neuron identity, a double-labeling for eGFP and HuC/D was performed. Indeed, some eGFP+ cells were also HuC/D+, indicating that these cells were early differentiating neurons (Fig. 16D). No eGFP-labeled cells were found between the electroporation site in the diencephalon and the stained cells in the midbrain after 4d incubation (Fig. 16B).

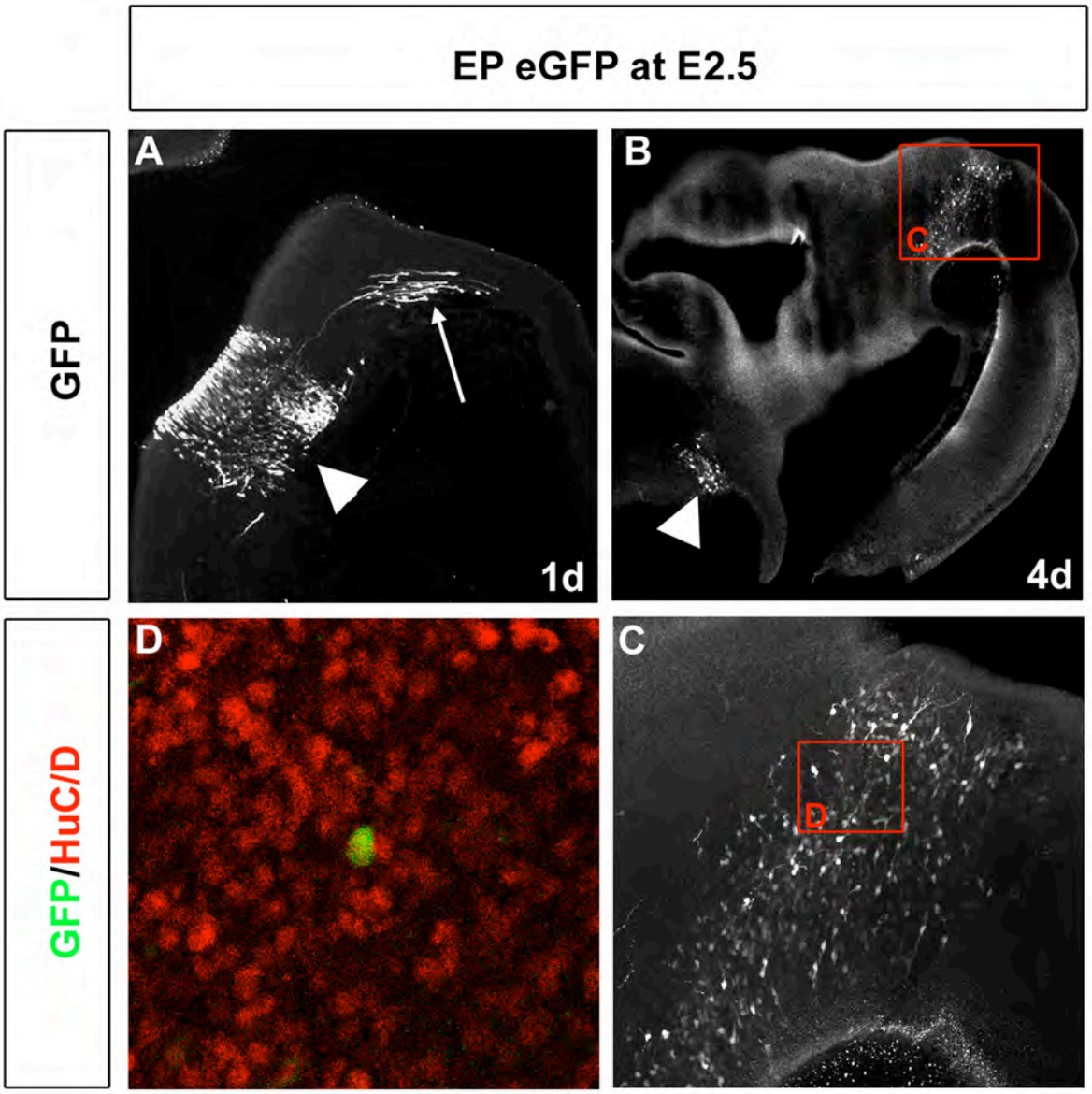

Figure 16: Evidence for a caudal migration of neuronal precursors *in vivo*. (A) Close-up view of a sagittal vibratome section of a chicken brain at E3.5 stained for GFP 1d after EP. The electroporation site in the diencephalon is clearly visible (arrowhead) and additionally, GFP stained nuclei and processes could be detected migrating towards the midbrain (arrow). (B) Sagittal vibratome section of a chicken brain at E6.5 4d after EP. GFP+ nuclei were detected in the ventral midbrain. The arrowhead indicates the electroporation site in the diencephalon. The red box marks the close-up of the region shown in (C). (C) GFP labeled nuclei displayed a neuron morphology. The red rectangle indicates the region shown in (D). (D) Close-up of a double labeled cell in the ventral midbrain for GFP (green) and HuC/D (red) taken with a laser scanning confocal microscope.

3.1.6 *Pitx3* induction in the midbrain probably follows a signaling gradient from the diencephalon

To reveal, if a progressive *de novo* induction of Pitx3 in the midbrain takes place *in vitro* experiments were performed. Therefore, explant cultures of a) entire anterior neural tube, b) midbrain only or c) forebrain only were done. If the induction/expression of *Pitx3* follows a signaling gradient emanating from a secondary organizer in the forebrain and/or the midbrain, this would be reflected by differences in the induction and/or expression of *Pitx3* between the explants.

Whole anterior neural tube, midbrain only and forebrain only explants were prepared from HH14 (E2.25) and HH16 (E2.5) chicken embryos. As *Pitx3* expression begins at stage HH16/17 in the diencephalon, the explants prepared from HH14 embryos should reveal whether *Pitx3* is induced under the culture conditions. Explants prepared from HH16 embryos could answer the question whether *Pitx3* is induced and/or maintained in the absence of forebrain and/or mid-/hindbrain tissue. *Pitx3* was not induced/expressed in isolated midbrain explants dissected at HH14/HH16 after 4d/5d of incubation, a stage corresponding to E6/6.5, when *Pitx3* expression was readily detected in the chicken embryo (n=7/8; Fig. 17C´, C´´). By contrast, *Pitx3* was induced/expressed in the majority of isolated forebrain explants (n=7/10; Fig. 17B´; B´´) and whole brain explants (n=7/8; Fig. 17A´; A´´).

In summary, *Pitx3* is induced/expressed in forebrain and whole brain explants, which suggests that signaling from the forebrain (e.g. ZLI) is required. *Pitx3* is not induced/expressed in midbrain explants, which indicates that signaling from the midbrain (e.g. MHB) is not sufficient for *Pitx3* induction or that intact brain tissue is required for a caudal migration of *Pitx3*+ precursors. Therefore, the diencephalon is needed for the induction/expression of *Pitx3* in the chicken embryo.

This suggests that *Pitx3* expression in the ventral midbrain of the chicken embryo may be due to: 1) a caudal migration of *Pitx3*+ postmitotic neuronal precursors born in the diencephalon and/or 2) a caudal *de novo* induction of *Pitx3* in the midbrain due to signals emanating from the forebrain.

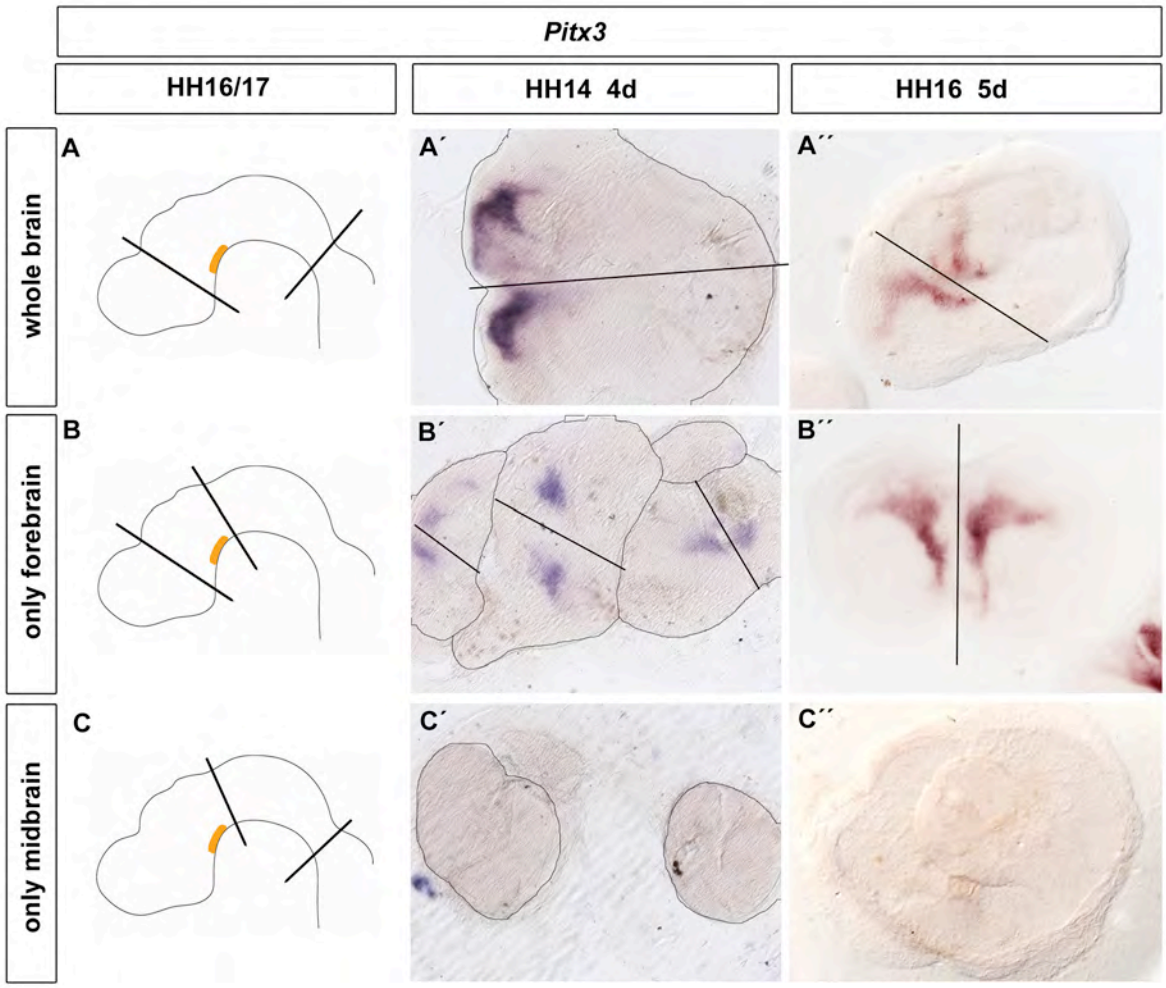

Figure 17: *Pitx3* is not induced in isolated chicken midbrain explants. (A-C) Schematic drawings of the E2.5 chicken brains. Black lines in (A-C) delimit the regions of the HH14-16 chicken neural tube used for preparation of whole brain (A´, A´´), forebrain only (B´, B´´) or midbrain only (C´, C´´) explants. (A´-C`) Explants dissected at HH14, incubated as whole brain (A´), forebrain (B´) or midbrain (C´) for 4d and stained with a probe for *Pitx3*. The whole brain (A´) and forebrain (B´) explants showed a clear staining for *Pitx3*, whereas the midbrain explants are devoid of *Pitx3* expression (C´). (A´´-C´´) Explants dissected at HH16, incubated as whole brain (A´´), forebrain (B´´) or midbrain (C´´) for 5d and stained for *Pitx3*. An induction of *Pitx3* was only detected in whole brain (A´´) and forebrain (B´´) explants, but not in midbrain explants (C´´). The black lines in (A´-C´´) indicate the ventral midline.

3.1.7 Analysis of the functional role of Pitx3 in the development of mesDA neurons in the chicken embryo

Given the striking correlation between Pitx3 expression and appearance of the other DA neuron markers, as assessed by the expression of *Th, Nr4a2* and *Lmx1b*, I suggested a role for Pitx3 in the early development of mesDA neurons in the chicken embryo. Therefore, the function of Pitx3 in the chicken embryo was analysed by electroporating morpholinos, dominant-active or dominant-repressive expression constructs, respectively.

3.1.7.1 Knock-down of Pitx3 expression in the chicken embryo using morpholinos

Morpholino oligos are short chains of about 25 so-called morpholino subunits. Each subunit is comprised of a nucleic acid base, a morpholine ring and a non-ionic phosphorodiamidate intersubunit linkage. Morpholinos act via a steric block mechanism and with their high mRNA binding affinity and specificity they either can block translation initiation in the cytosol (by targeting the 5' UTR through the first 25 bases of coding sequence), they can modify pre-mRNA splicing in the nucleus (by targeting splice junctions) or they can block miRNA activity. It has already been shown that microinjection or electroporation of morpholino oligos into the embryos of frogs, zebrafish, chicken, sea urchins and other organisms leads successfully and specifically to a knock-down of the expression of targeted genes (Heasman et al., 2000; Nasevicius and Ekker, 2000; Kos et al., 2003; reviewed by Corey and Abrams, 2001).

Therefore, two morpholinos were designed: one which blocks the translation and one which modifies the splicing of the *Pitx3* mRNA (Fig. 18), leading to no or a truncated Pitx3 protein. As a control, a standard control oligo was used designed and offered by GeneTools, LLC, which has no target gene or biological activity. The morpholinos were electroporated into the neural tube of HH10-12 (E1.5) chicken embryos. After an incubation of 6 days *in ovo,* the embryos were fixed and analysed for the expression of different mesDA marker genes. However, a difference in the expression pattern was not detected for any of the analysed genes. Expression of *Th, En1* and *Lmx1b* was unaffected after the electroporation of the splice- or start-morpholinos (Fig. 19B-D and data not

shown; compare with Fig. 10). The expression of *Pitx3* was not affected (Fig. 19A and data not shown; compare with Fig. 10), although it may have been that the *Pitx3* mRNA should get unstable after the morpholino treatment. Therefore, a failure in the effectiveness of the morpholinos can not be excluded.

catctctgttttccagccaggaaacccagaaaggatccgaggatcacacagggatcctcag
ctttccctaggcctctcctcaggggctggccagtccctttcacacggatgggaagaggtc
acgagcacagatccaactccctaacaaccccgtgcctctttttcaggttcctccatcacca
ccacccattcctgccccATGGATTTCAACCTGCTGGCGGACGCGGAGGCTCGCAGCCCAGC
CCTGTCCCTCTCAGATTCCGGCACCCCCCAGCACGAGCACAGCTGCAAGGGGCAGGACCAT
AGCGgtgagtgtgaggtcccacgctcctg.....tctcccatctttgttttgcata
cagATACTGAGAAGTCCCAACAGAACCAGACAGACGACTCCAACCCCGAGGACGGCTCACT
CAAGAAGAAGCAGCGGAGGCAGCGGACTCACTTCACCAGCCAGCAGCTCCAGGAGCTGGAA
GCCACGTTCCAGAGGAACCGCTACCCCGACATGAGCACCAGGGAGGAGATCGCGGTCTGGA
CCAACCTGACAGAGGCACGAGTGCGGgtaggactgtggccctgtgcacacc.....
tctccacctctctcctttcttacagGTCTGGTTCAAAAACCGCAGGGCTAAGTGGAGGAAA
CGGGAGAGGAACCAACAGGCTGAGCTCTGCAAGAACAGCTTTGGAGCCCAGTTCAACGGAC
TGATGCAGCCCTACGACGACATGTATTCTAGCTATTCCTACAACAACTGGGCCACCAAAGG
GCTCGCCACCAGCCCGCTCTCAGCCAAGAGCTTCCCATTCTTCAACTCCATGAATGTCAGC
CCCCTCTCCTCCCAGCCCATGTTCTCCCCACCCAGCTCCATCGCCTCGATGACCATGCCCT
CATCCATGGTCCCTTCTGCAGTGACGGGCGTCCCGGCCTCCAGCCTCAACAACCTGGGAAA
CATCAACAATCTGAATAGCCCGAGCCTCAACTCCGCCGTCTCATCCAGTGCCTGTCCTTAT
GCCTCCACAGCCAGCCCCTACATGTACAGGGACACGTGCAACTCCAGCCTGGCAAGCCTGA
GGCTGAAGGCCAAGCAGCACGCCAACTTCACTTACCCAGCGGTGCAGACGGCAGCTTCCAA
CCTGAGCCCTTGCCAATACGCTGTGGACAGGCCTGTATGAagtgctgctctctgctaactg
gggacttgaccttagcctgacacaaggagacctttagccctacaacagcgtacatcctgca
gagtgagagtggaagcaagagagagcaaaagagcatgagagagagagagcacacagcaagc
aggcagatggacctctatgaagatttgtctaactatcgtatggtgaattttgactgt...

Figure 18: Coding sequence of the chicken Pitx3 gene (ENSGALG5613) used for the morpholino design. The green color assigns the 5´UTR and 3´UTR, the black color assigns the exons and the blue color assigns the introns of the Pitx3 gene. The start codon is highlighted in red. The underlined sequence was used as a target sequence for the morpholinos: the start-morpholino covering the first 25 basepairs of the coding sequence to block translation, the splice-morpholino covering the 14 last basepairs of the first intron and the 11 first basepairs of the second exon to modify the splicing.

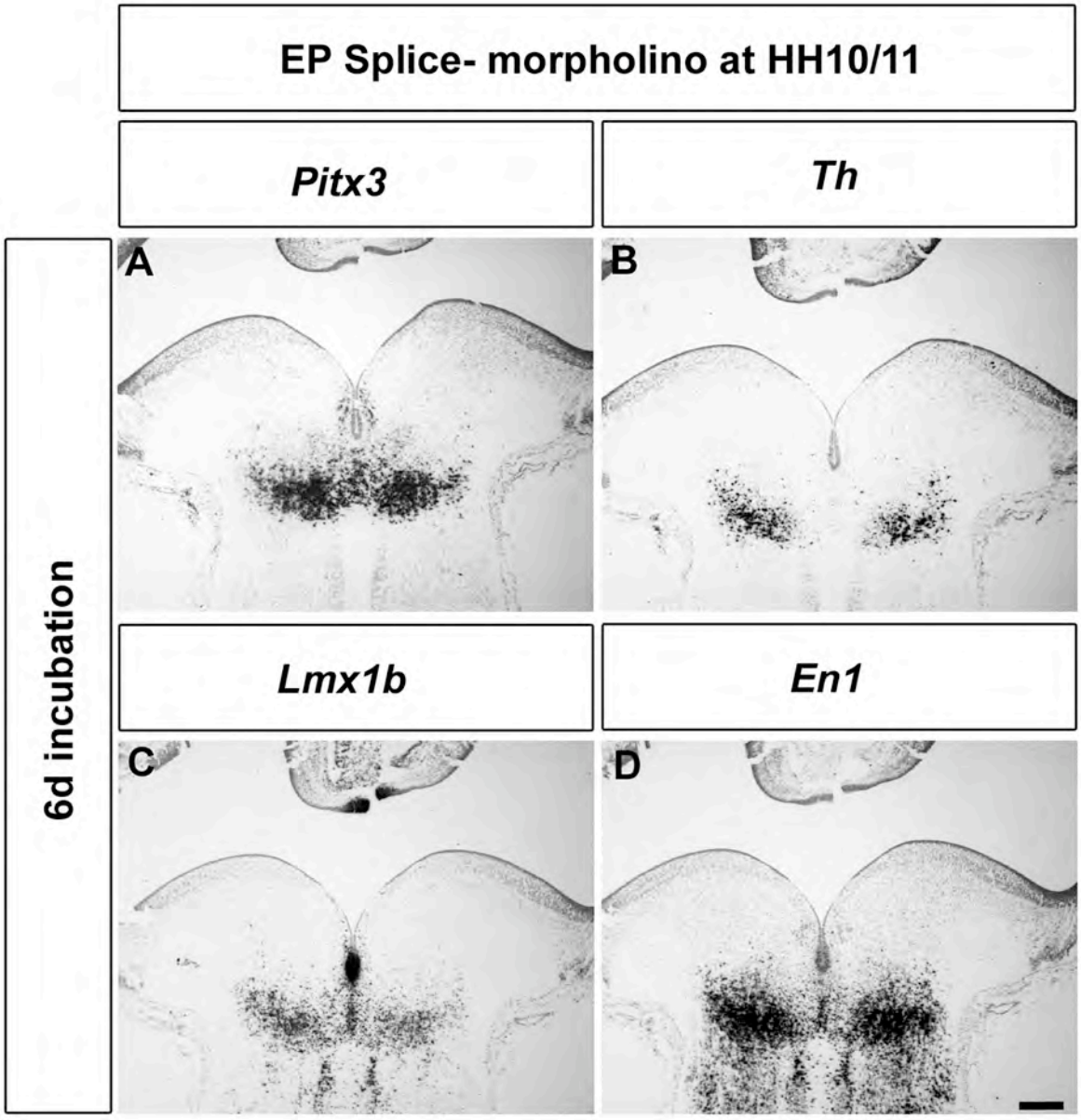

Figure 19: Morpholino knock- down of Pitx3 did not disturb mesDA neuron development in chicken embryos. Consecutive coronal sections of a brain electroporated with a splice-morpholino, incubated for 6 days and hybridised with probes for *Pitx3* (A), *Th* (B), *Lmx1b* (C) and *En1* (D). No differences in the expression pattern of these mesDA marker genes was observed (compare with Fig. 9). Scale bar: 200 μm.

3.1.7.2 Activator or repressor function of Pitx3 in the chicken embryo

Another approach to test Pitx3 function in the chicken embryo brain was the construction of two different expression plasmids. Here the homeodomain (HD) of the Pitx3 gene was fused in frame to two transcriptional activator (TA2) domains of the VP16 enhancer of the Herpes simplex virus protein (Baron et al., 1997) or to the *Drosophila* Engrailed Repressor domain (EnR) (Bellfroid et al, 1996), respectively. The HD will mediate the DNA binding properties of Pitx3 to specific sequences in target genes. Fused to a transactivator domain, the overexpression of this construct (HDPitx3-TA2) would cause a phenotype if Pitx3 is a repressor of target genes, because it would now transactivate them. On the other hand, if the HD of Pitx3 is fused to a repressor domain (HDPitx3-EnR) and Pitx3 would normally act as an activator, the overexpression would then cause a phenotype by now repressing target genes.

3.1.7.2.1 Injection of HDPitx3-TA2 and HDPitx3-EnR into zebrafish embryos

The functionality of the constructs was tested in the zebrafish system. Therefore, capped mRNA synthesized from the constructs was injected into zebrafish embryos at the one-cell stage and incubated for 3 days. To visualize the successful injection, a plasmid encoding for a membrane-bound red fluorescence protein (mRFP) was co-injected.

Embryos injected with the HDPitx3-EnR construct showed a lethal phenotype. All zebrafish embryos injected were already dead after 24 hours (data not shown). This observation corresponds well to results published by Faucourt et al. (2001). These authors showed that the coding sequence of the Xenopus *Xpitx2b* gene fused to the EnR domain specifically interferes with the process of endoderm specification and therefore leads to an early gastrulation phenotype in Xenopus embryos. Since the paired-like homeodomain of the Pitx3 and Pitx2 proteins is highly conserved, it is very likely that the HDPitx3-EnR protein would also target Pitx2 and/or Pitx1 target genes and thus cause a similar phenotype as described by Faucourt et al. (2001).

Embryos injected with the HDPitx3-TA2 construct showed variable phenotypes (Fig. 20A, B): 20% showed a mild phenotype with slightly smaller eyes and disrupted pectoral fin development (Fig. 20C, arrowheads). 20% of the embryos showed a strong eye

phenotype. The embryos developed only one eye (Fig. 20D, arrowhead). A very severe phenotype was observed in 10% of the injected embryos. The embryos had no eyes at all and a severe body axis deviation (Fig. 20E). The remaining 50% of the injected embryos showed no apparent phenotype. A similar result concerning the phenotypes was observed in zebrafish embryos injected with a morpholino to knock-down expression of the zebrafish Pitx3 protein, meaning that 50% of the injected embryos had smaller eyes and mild to moderate body axis deviation (Shi et al., 2005). The results obtained in zebrafish

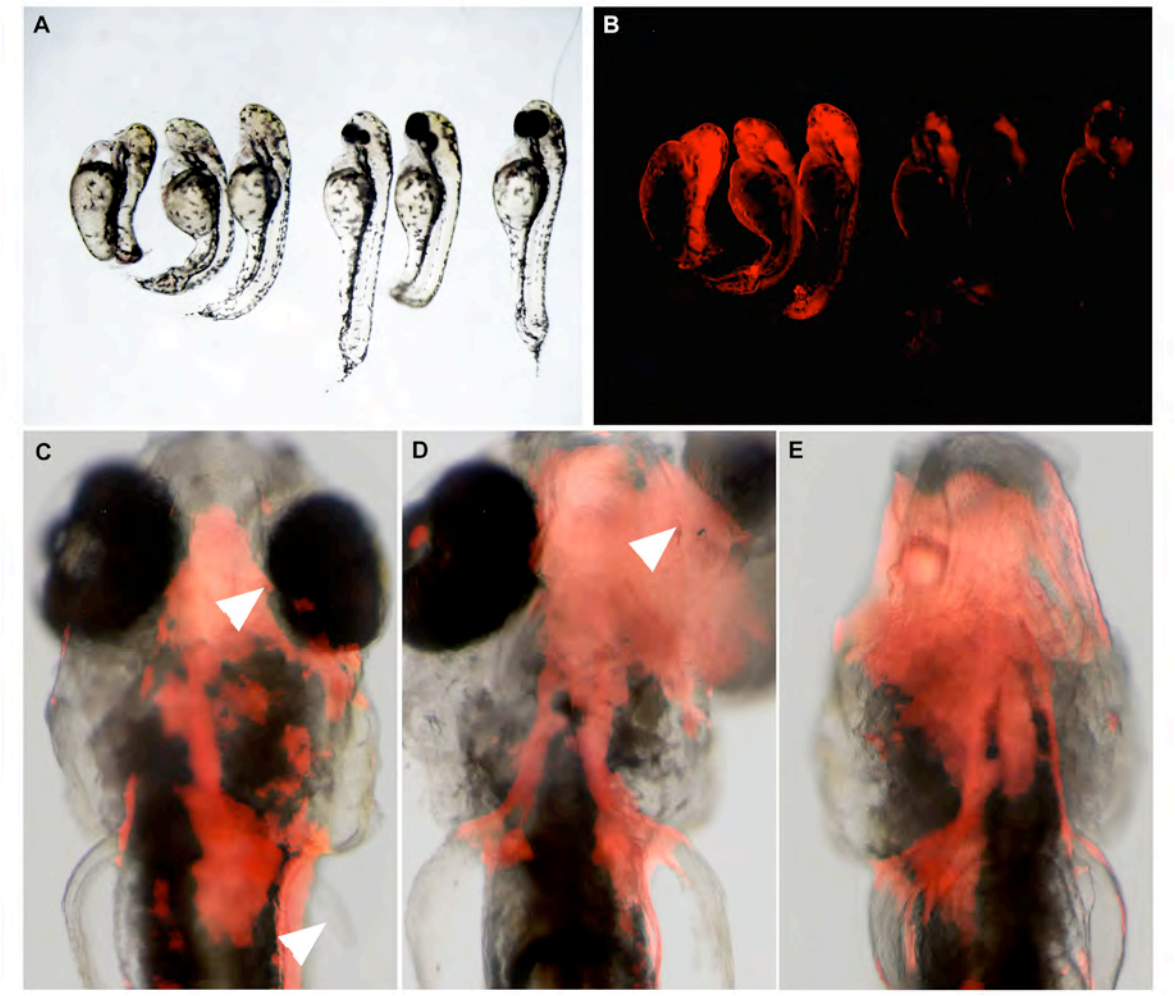

Figure 20: Injection of the HDPitx3-TA2 construct leads to a similar phenotype using the Pitx3 morpholino in zebrafish embryos. (A) Overview of the different phenotypes observed 3d after HDPitx3-TA2 injection varying from mild (right) to severe (left) developmental defects. (B) Fluorescence image of (A) showing the co-injected mRFP. (C) Zebrafish embryo showing a mild phenotype with a smaller eye and disrupted pectoral fin (arrowheads). (D) 20% of the embryos missed one eye (arrowhead). (E) In 10% of the embryos a strong phenotype with no eyes and severe body axis deviation was visible.

embryos indicated that both expression constructs are functional and can be used for chicken *in ovo* electroporation. In addition, the outcome of the HDPitx3-TA2 injection indicates that Pitx3, at least in the zebrafish, acts as a transcriptional repressor as the observed phenotype is identical to the one detected after morpholino knock-down.

3.1.7.2.2 Electroporation of HDPitx3-TA2 and HDPitx3-EnR into chicken embryos

After electroporation of the HDPitx3-TA2 and HDPitx3-EnR constructs into the ventral midbrain of chicken embryos at stage HH10-12, the eggs were reincubated for 6 days. This time-point was chosen because at this stage *Th* expression can be detected (see Fig. 10B, B´). For both constructs – HDPitx3-TA2 and HDPitx3-EnR – the mesDA marker genes *Th*, *Pitx3*, *Nr4a2* and *Lmx1b* were expressed in a normal pattern (Fig. 21A-D and data not shown; compare to Fig. 10).

Taken together, from these results I inferred that the expression constructs as well as the morpholinos were diluted to much after an incubation of 6d because of the massive growth of the embryo during early development. Therefore, it is more likely that the electroporated chicken embryos showed no phenotype due to technical problems rather than no function of Pitx3 during early development of mesDA neurons in the chicken embryo. For future long-term incubation experiments it is feasible to use a transposon-mediated gene transfer technique, which enables an electroporated gene to be stably integrated into the chicken genome (Sato et al., 2007).

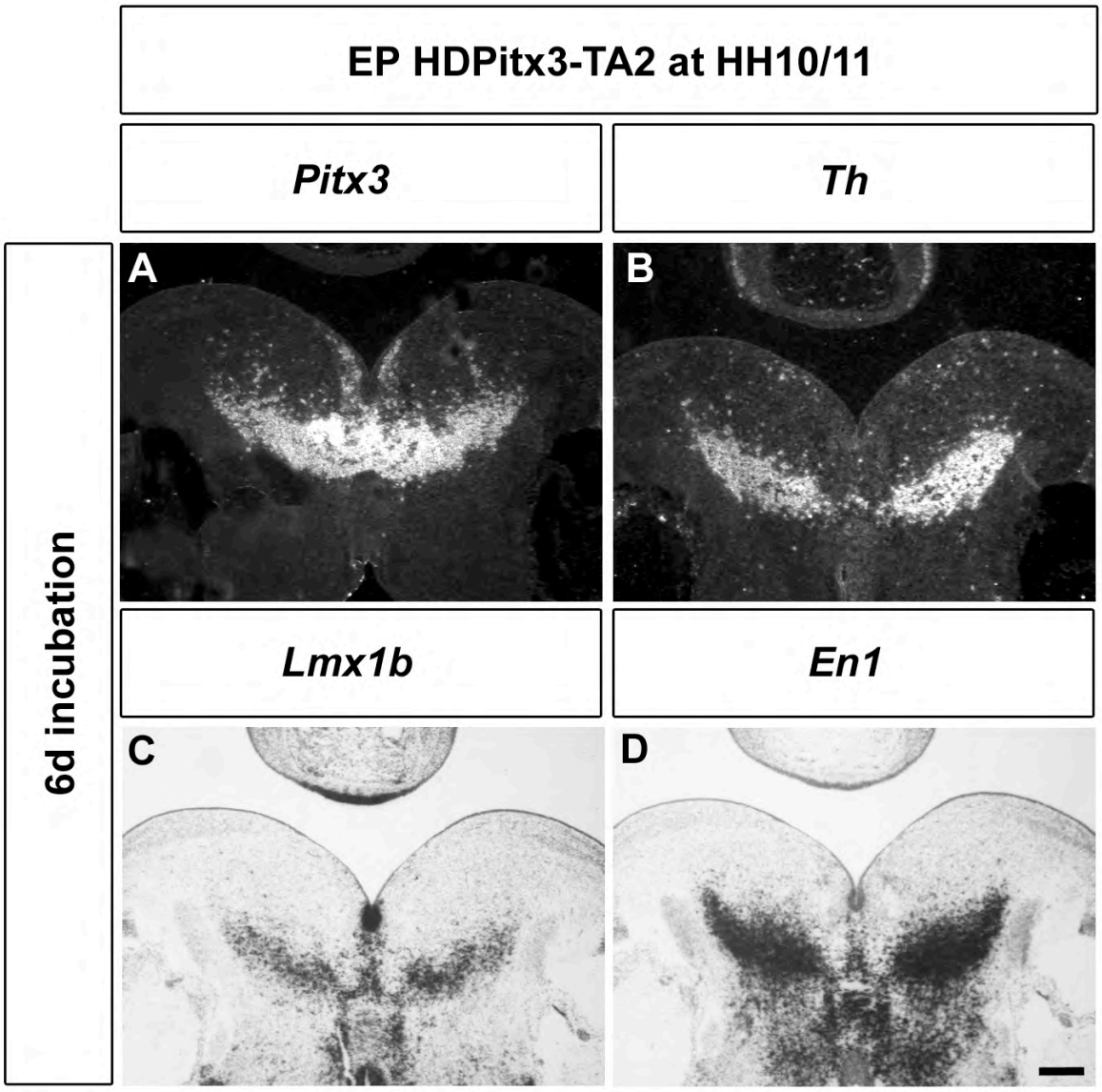

Figure 21: Normal development of mesDA neurons after electroporation of the HDPitx3-TA2 activator construct. Consecutive coronal sections of an embryonic chicken brain electroporated with the HDPitx3-TA2 activator construct, incubated for 6 days and hybridised with probes for *Pitx3* (A), *Th* (B), *Nr4a2* (C) and *Lmx1b* (D).

3.1.8 Comparison of the *Pitx3* expression in mouse and chicken embryos

The striking differences between the spatio-temporal expression of chicken (*c*)*Pitx3* and the expression of mouse (*m*)*Pitx3* reported by Smidt et al. (1997) led us to reexamine the expression of *mPitx3* during mouse embryonic development. Although no expression of *mPitx3* was detected in the E11.5/E12.5 embryonic brain other than the ventral midbrain/cephalic flexure (Smidt et al., 1997), a novel expression domain of *mPitx3* was detected in the lateral tectum of the E13 mouse embryo (Fig. 22A´, arrowhead). This dorso-lateral expression domain of *mPitx3* becomes even more pronounced at E13.5 (Fig. 22B´, arrowhead). At E14.5, the dorso-lateral expression of *mPitx3* appears to be confined

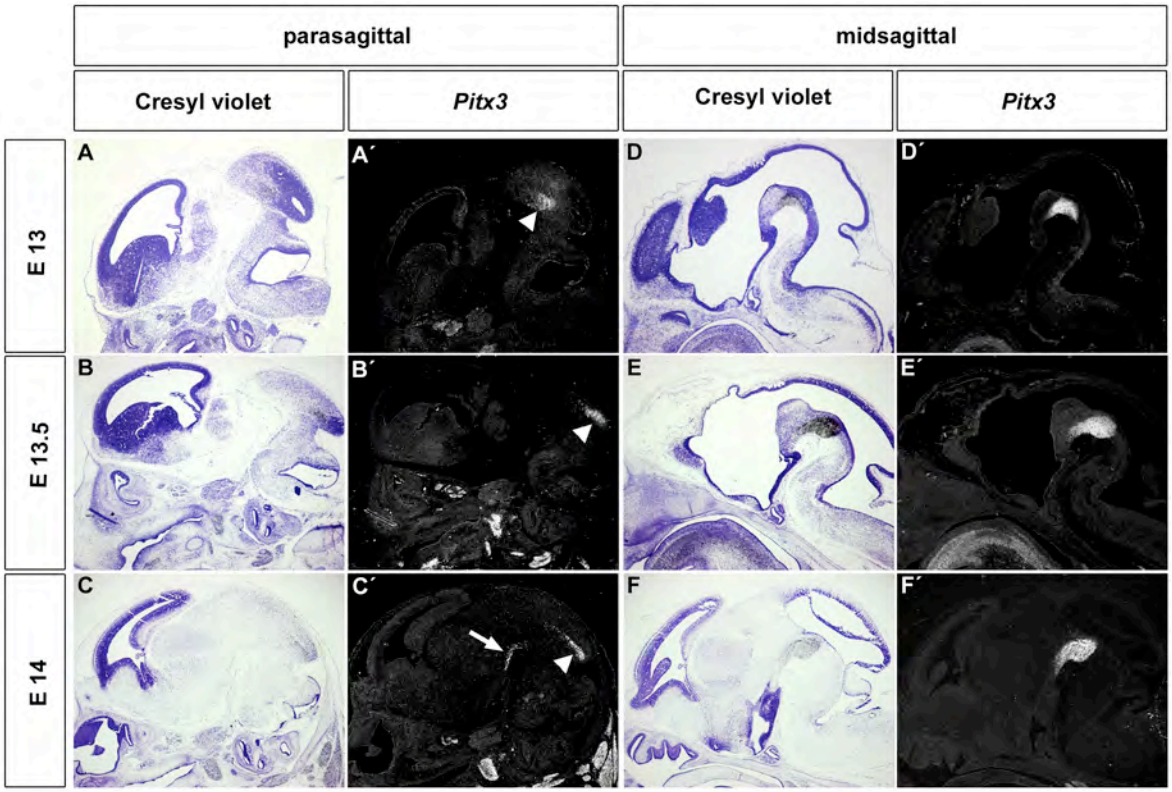

Figure 22: Novel expression domains of mouse *Pitx3* in the developing mouse embryo.(A-F) Brightfield images of (A´-F´). At E13, a dorsal expression domain of *mPitx3* was detected in the tectum on a parasagittal section (A´, arrowhead). Ventral midbrain expression of *mPitx3* (as described by Smidt et al., 1997) was detected on a midsagittal section (D, D´). From E13.5 on, the dorso-lateral expression domain of *mPitx3* appears to be restricted to posterior tectum (B´, arrowhead), whereas the medial expression domain was broadened (E´). At E14, the dorso-lateral expression of *mPitx3* appears to be restricted to the caudal tectum (C´, arrowhead) and a ventral diencepahlic expression domain of *mPitx3* was first visible (C´, arrow).

mostly to the caudal tectum, the presumptive inferior colliculus (IC) (Fig. 22C´, arrowhead). Furthermore, an additional *mPitx3* expression domain extending into the ventral diencephalon became discernable at this stage (E14.5) in the mouse embryo (Fig. 22C, arrow). The expression of *mPitx3* in the cephalic flexure was detected throughout all examined stages, from E13 until E14.5 (Fig. 22D-F´).

The ventral diencephalic and posterior tectal (presumptive IC) expression domains of *mPitx3* are clearly discernable in the E15.5 and still detectable in the E18.5 mouse embryo (shortly before birth) (Fig. 23B, B´). At E 18.5, however, the dorsal *mPitx3* expression appears to be confined to a small domain in the IC (Fig. 23B´). To establish whether these novel *mPitx3*+ domains also express *mTh* and may thus correspond to DA populations other than the mesDA neurons, an ISH for *mTh* was done on consecutive sagittal sections of E15.5 and E18.5 mouse embryonic brains. A clear overlap of the *mTh* and *mPitx3* expression domains was detected in the diencephalon of the E15.5 (Fig. 23D) and E18.5

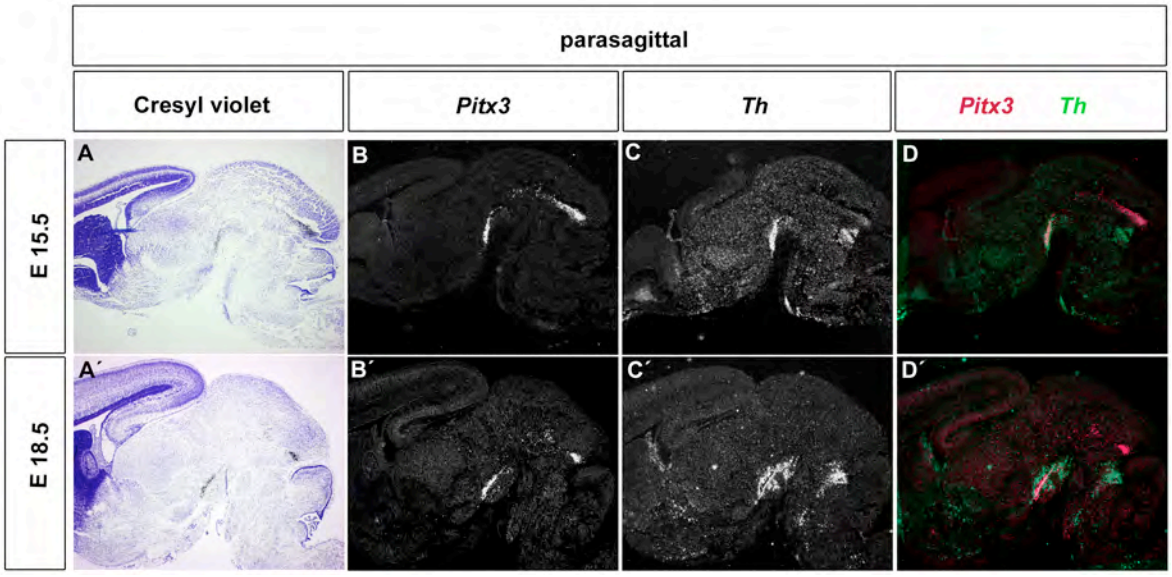

Figure 23: Novel mouse Pitx3 expression domains in tectal and diencephalic regions. (A, A`) Brightfield images of (B, B´). (A-D) Consecutive parasagittal sections of an E15.5 mouse brain hybridised with probes for *Pitx3* (B) and *Th* (C). (D) Pseudocolored overlay of *Pitx3* (red) anf *Th* (green). Overlapping regions appear in yellow. (A´-D´) Consecutive sagittal sections of an E18.5 mouse brain hybridised with probes for *Pitx3* (B´) and *Th* (C´). (D´) Pseudocolored overlay of *Pitx3* (red) and *Th* (green) showed a clear overlap of the two expression domains in the diencephalon (yellow).

(Fig. 23D´) mouse embryo, but not in the posterior tectum/IC. This indicated that *mPitx3* also has a diencephalic expression domain that has not been described so far.

The strinking difference between the chicken and the mouse *Pitx3* expression is the temporal onset: in the chicken embryo, *cPitx3* is first detected in the ventral diencephalon and later induced in the ventral midbrain. In the mouse embryo, *mPitx3* is first expressed in the ventral mesencephalon and later also in a ventral diencephalic domain.

3.2 Wnt signaling in mesDA neuron development in the chicken embryo

The secreted glycoprotein Wnt1 has been shown to play a crucial role in the development of mesDA neurons in the mouse (Prakash et al., 2006). To investigate whether Wnt signaling is also required in the development of mesDA neurons in the chicken embryo, first an expression analysis was performed for several *Wnt* genes during embryonic development to determine whether these *Wnts*, and *Wnt1* in particular, are expressed in a similar pattern in the chicken as in the mouse embryo. Second, overexpression of *Wnt* in the chicken embryo was used to assess whether the Wnt-controlled induction of mesDA neurons is a conserved mechanism between chicken and mouse. Furthermore, the electroporation technique in the chicken embryo provides a strong tool to reveal factors acting upstream and downstream of Wnt.

3.2.1 Expression analysis of nine *Wnt* genes in the anterior neural tube of the early chicken embryo

3.2.1.1 *Wnt1* is not expressed in the cephalic flexure of the chicken embryo

Wnt1 is strongly expressed in the roof plate of the anterior neural tube and in a ring encompassing the MHB, but it is not expressed in the cephalic flexure of the chicken embryo, in contrast to the mouse embryo (Fig. 24B, B´ and data not shown; Parr et al., 1993). Therefore, an expression analysis for nine *Wnt* genes was done in the developing chicken embryo focussing on the ventral part of the anterior neural tube. The nine analysed *Wnt* expression domains were compared with that of the paired-like homeodomain transcription factor *Pitx3*, the earliest marker for mesDA neurons in the chicken embryo (Fig. 24A, A´; Fig. 25A, A´). The restriction to nine out of 19 *Wnt* genes was made because of the limited sequence data available in chicken. A previous study on the expression pattern of *Wnt* genes in the chicken embryo concentrated on dorsal structures of the neural tube (Hollyday et al., 1995). *Wnt3a* mRNA was present in the dorsal midline continously from the diencephalon to the metencephalon (Fig. 24C and data not shown).

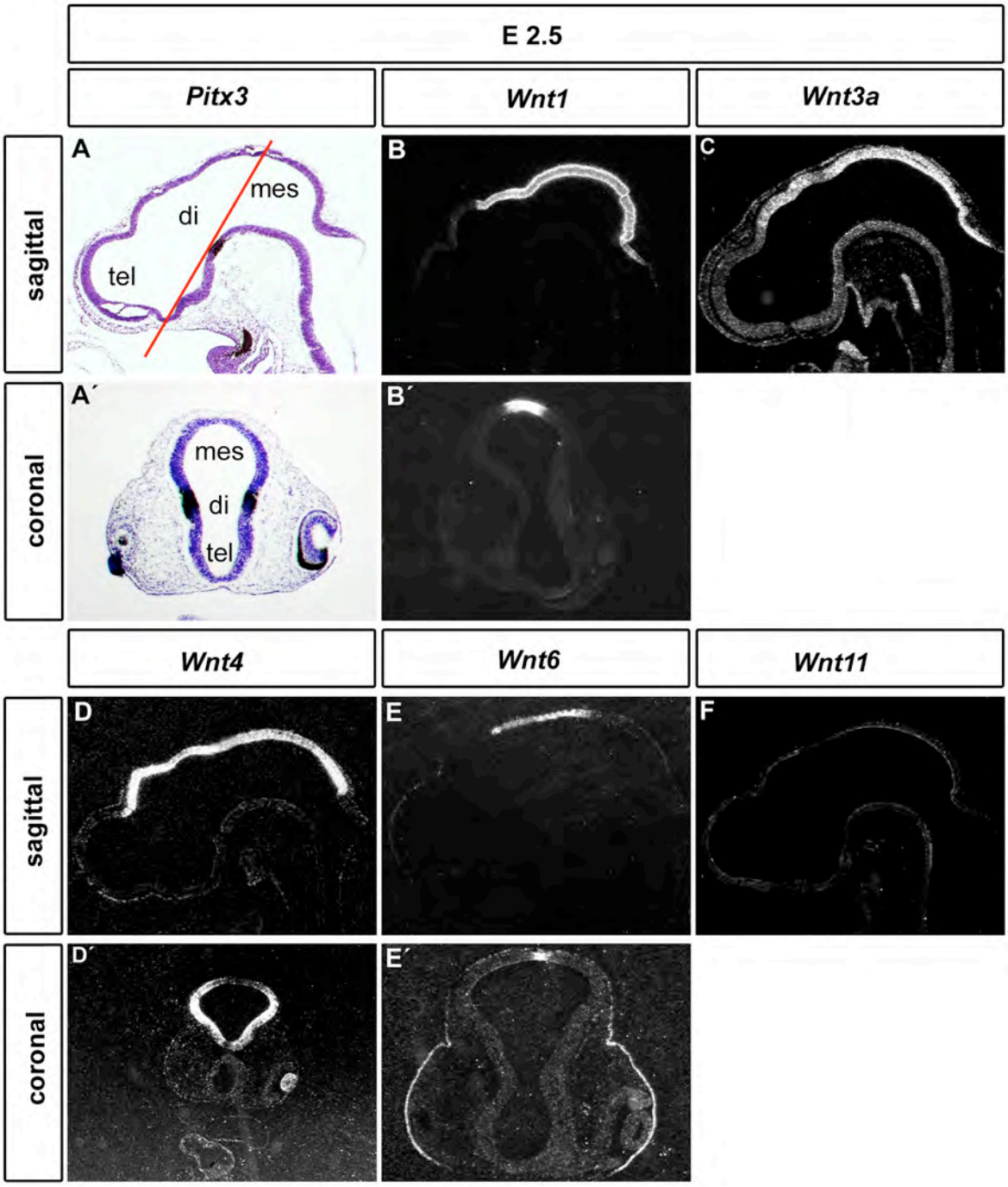

Figure 24: Expression of five *Wnt* genes in the dorsal neural tube of E2.5 chicken embryos. (A-F) Midsagittal sections of E2.5 chicken embryos. Brightfield image of a sagittal (A) and coronal (A´) section hybridised with a probe for *Pitx3*. The red line in (A) indicates the level of the coronal sections (A´-E´). The darkfield images were taken from the corresponding sections hybridised with probes for *Wnt1* (B, B´), *Wnt3a* (C, C´), *Wn4* (D, D´), *Wnt6* (E, E´) and *Wnt11* (F). All *Wnt* genes shown here, except *Wnt11*, are expressed in the dorsal di- and/or mesencephalon, but not in the cephalic flexure overlapping with the *Pitx3* expression. Abbreviations: di, diencephalon; mes, mesencephalon; tel, telencephalon.

Strong expression of the *Wnt4* gene was observed in the dorsal and lateral walls of the di- and mesencephalon, excluding the roof and floor plate (Fig. 24D, D´). Transcripts of *Wnt6* were detected in the roof plate of the posterior di- and anterior mesencephalon in line with a previous study (Fig. 24E, E´; Schubert et al., 2002). *Wnt11* was not expressed in the anterior neural tube of the chicken embryo at this stage (Fig. 24F).

Nevertheless, expression of four *Wnt* genes was detected in the cephalic flexure at E2.5: *Wnt5a, Wnt5b, Wnt7a,* and *Wnt9a* (formerly known as Wnt14) (Fig. 25B-E; B´-E´), as partly reported before (Hollyday et al., 1995; Dealy et al., 1993). The *Wnt5a* gene showed expression in the entire ventral cephalic flexure across the MHB extending into the hindbrain (Fig. 25B). In the diencephalon, *Wnt5a* was also expressed in the dorsal midline of the anterior diencephalon (Fig. 25B). In the D/V axis, *Wnt5a* transcripts were detected in the floor and basal plate of the di- and mesencephalon (Fig. 25B´). *Wnt5b* was expressed in the floor and basal plate of the cephalic flexure but it appeared much weaker than the *Wnt5a* expression (Fig. 25C,C´). In addition, *Wnt5b* was also expressed in the roof plate of the most caudal part of the midbrain (Fig. 25B). *Wnt7a* transcripts were detected in the ventral mid- and hindbrain (Fig. 25D). The coronal section showed that *Wnt7a* was strongly expressed in the lateral walls of the midbrain, excluding the roof plate (Fig. 25D´). *Wnt9a* was strongly expressed in the roof plate of the tel-, di- and mesencephalon (Fig. 25E, E`). Ventrally, *Wnt9a* expression was detected in the floor plate of the diencephalon and anterior mesencephalon (Fig. 25E, E´) overlapping with the *Pitx3* expression domain.

In summary, four of the nine *Wnt* genes *Wnt5a, Wnt5b, Wnt7a* and *Wnt9a*, showed an overlap with the *Pitx3* expression domain and can therefore be good candidates for having taken over the function of Wnt1. Until now, a total of 19 *Wnt* genes have been descibed in the human and mouse genomes, which means that more candidates are possible in the chicken embryo. As stated before, not all *Wnt* gene sequences are yet available from the chicken genome. However, I focused on the *Wnt9a* gene for a functional study, because *Wnt5a* has been described as a non-canonical *Wnt* (reviewed by Wodarz and Nusse, 1998), whereas *Wnt9a* acts through the canonical pathway (Guo et al., 2004). It was suggested that *Wnt1* also acts through the canonical pathway to direct mesDA neuron development in the mouse embryo (Prakash et al., 2006; Castelo-Branco et al., 2004). *Wnt5b* and *Wnt7a* may have been other reasonable candidates, but they are only very weakly expressed in the

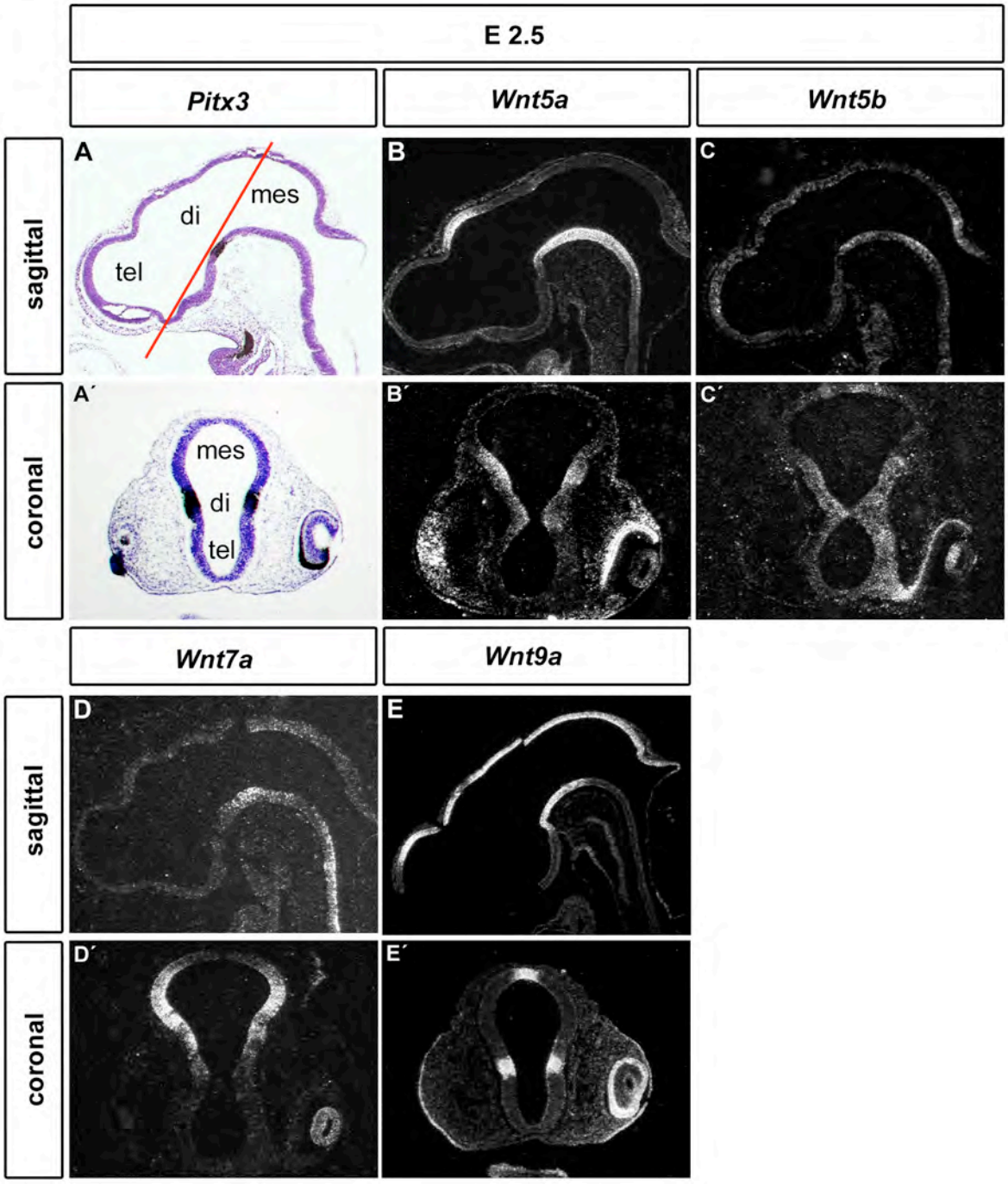

Figure 25: Expression of four *Wnt* genes in the dorsal and ventral anterior neural tube of E2.5 chicken embryos. (A-E) Sagittal sections of E2.5 chicken embryos. (A) Brightfield image of a sagittal section hybridised with a probe for *Pitx3*. The red line indicates the sectioning level of the coronal sections. The darkfield images were taken from corresponding sections hybridised with probes for *Wnt5a* (B), *Wnt5b* (C), *Wnt7a* (D) and *Wnt9a* (E). (A´-E´) Coronal sections of E2.5 chicken embryos. (A´) Brightfield image of a coronal section hybridised with a probe for *Pitx3*. The darkfield images show coronal sections hybridised with probes for *Wnt5a* (B´), *Wnt5b* (C´), *Wnt7a* (D´) and *Wnt9a* (E´). Abbreviations: di, diencephalon; mes, mesencephalon; tel, telencephalon.

rostral cephalic flexure compared to *Wnt9a*. Therefore, the *Wnt9a* gene appeared to be the best candidate and its expression in the chicken embryo was analysed more precisely.

3.2.1.2 Expression of *Wnt9a* and *Pitx3* at E3.5 and E5 of chicken embryonic development

The expression analysis of *Wnt9a* was further extended to see whether *Wnt9a* was still expressed in the same domain as *Pitx3* at later stages of development. At E3.5, *Wnt9a* was expressed throughout the whole neuroepithelium in the cephalic flexure (Fig. 26D, E), overlapping with the *Pitx3* expression domain in this region (Fig. 26A, B). At E5, the *Pitx3* and *Wnt9a* expression domains had expanded caudally abutting the MHB (Fig. 26C, F; arrowheads). While at this stage *Wnt9a* appeared to be expressed in the SVZ and VZ (Fig. 26F), the expression of *Pitx3* appeared to be restricted to the MZ of the ventral midbrain (Fig. 26C).

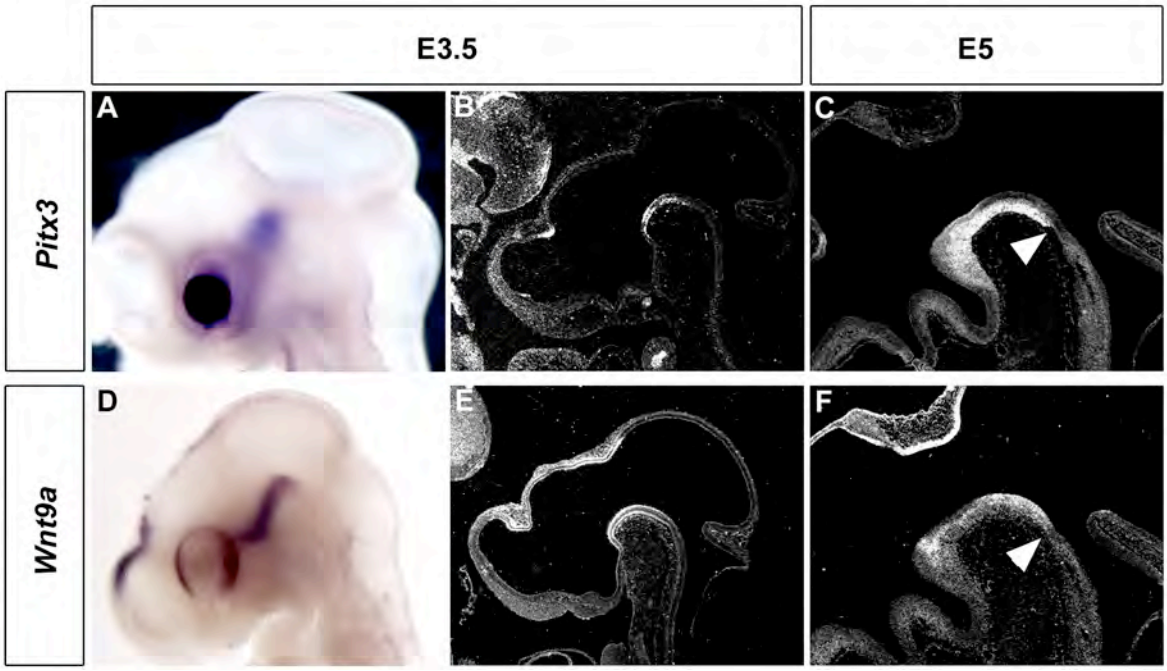

Figure 26: Coexpression of *Pitx3* and *Wnt9a* in the cephalic flexure of the chicken embryo at E3.5 and E5. (A, D) Whole mount ISH on E3.5 chicken embryos with probes for *Pitx3* (A) and *Wnt9a* (D). Midsagittal sections of E3.5 (B, E) and E5 (C, F) chicken embryos hybridised with probes for *Pitx3* (B, C) and *Wnt9a* (E, F). Arrowheads in (C, F) point at MHB.

Taken together, the *Wnt9a* expression strongly correlates with the *Pitx3* expression in the cephalic flexure also at later stages, shortly before mesDA neurons are first localized in the ventral midbrain.

3.2.1.3 *mWnt9a* expression in the anterior neural tube of the developing mouse embryo

As another pre-requisite for chicken *Wnt9a* being a good candidate to substitute for *Wnt1* function in the chicken embryo, it is important to know whether the *mWnt9a* expression domain shows redundancy with or exclusiveness of the *mWnt1* expression domain. As to date the expression pattern of *mWnt9a* in the CNS of the mouse embryo was not known, it was assessed at different stages of development. Notably, expression of *mWnt9a* was found in the roof plate of the fore-, mid- and hindbrain and also weakly in the ventral

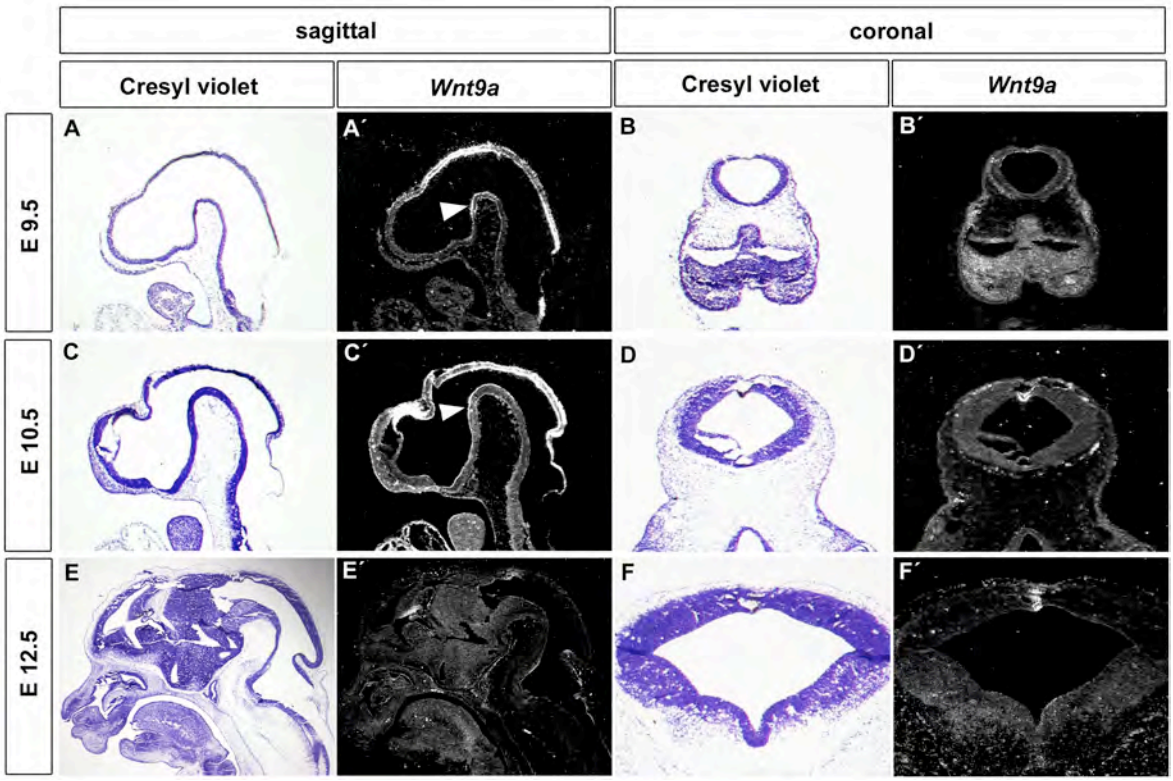

Figure 27: Expression of *Wnt9a* in the anterior neural tube of the mouse embryo during early development. (A-F) Brightfield images of (A´-F´). (A´-B´) At E9.5, *mWnt9a* was expressed in the roof plate of the fore-, mid- and anterior hindbrain and in a ventral diencephalic domain (arrowheads). (C´-D´) One day later, at E 10.5, there was still strong expression of *mWnt9a* in the roof plate of the fore-, mid- and hindbrain, whereas the diencephalic expression domain was weak. (E´-F´) At E12.5, the only detectable expression domain of *mWnt9a* was in the roof plate.

diencephalon of the mouse embryo at E9.5 (Fig. 27A, A´, B, B´; arrowhead). The *mWnt9a* expression pattern at this stage in the mouse resembles the expression of *cWnt9a* in the chicken embryo at the comparable stage E2.5 (Fig. 27A´; compare with Fig. 25E), although the ventral expression of *mWnt9a* in the mouse embryo appeared much weaker. One day later, at E10.5, a strong *mWnt9a* signal was still detected in the dorsal midline of the neural tube (Fig. 27C, C´, D, D´). The expression domain of *mWnt9a* in the ventral neural tube became very weak at this stage (Fig. 27C´, arrowhead) and was absent at E12.5 (Fig. 27E, E´, F, F).

In summary, *mWnt9a* expression in the mouse correlates only at E9.5 with the chicken *Wnt9a* expression. *mWnt9a* does not appear to play a role in mesDA development in the mouse embryo, as the expression domain of the early marker gene *mAldh1a1* is found much more caudally at E9.5/10.5. Thus, I concluded that *cWnt9a* may have substituted for *Wnt1* function in the chicken embryo based on its expression pattern.

3.2.2 The functional role of Wnt9a in the development of mesDA neurons in the chicken embryo

3.2.2.1 Electroporation of Wnt9a led to the induction of *Lmx1a* after 1d

To elucidate the role of Wnt9a in the development of mesDA neurons in the chicken embryo, the cDNA of the chicken *Wnt9a* gene was electroporated into the midbrain of chicken embryos. All electroporations were done at stage HH10-12 (E1.5). The successful electroporation was confirmed by *Wnt9a* or *GFP* expression, as the expression vector used possessed an internal ribosomal entry side (IRES)-GFP generating a bicistronic message (Krull, 2004). After an incubation of 1 day (1d), the expression of different genes was analysed. As it has been shown recently that Lmx1a is sufficient to induce mesDA neurons in the ventral midbrain of chicken embryos (Andersson et al., 2006b), the Wnt9a electroporated chicken embryos were analysed for the induction of *Lmx1a*. Normally, *Lmx1a* is expressed in the floor plate of the midbrain and caudal diencephalon (Andersson et al., 2006b). After the Wnt9a electroporation, an ectopic induction of *Lmx1a* was detected in the lateral midbrain (n=1/1; Fig. 28C, arrows). To confirm that the induction is not caused by a change in D/V patterning, *Shh* detection was used as a control, which is

expressed in the floor and basal plate of the midbrain at this stage. However, the *Shh* expression was not changed (Fig. 28D). None of the mesDA marker genes, like *Pitx3, Nr4a2* or *Lmx1b*, were induced 1d after Wnt9a electroporation (data not shown).

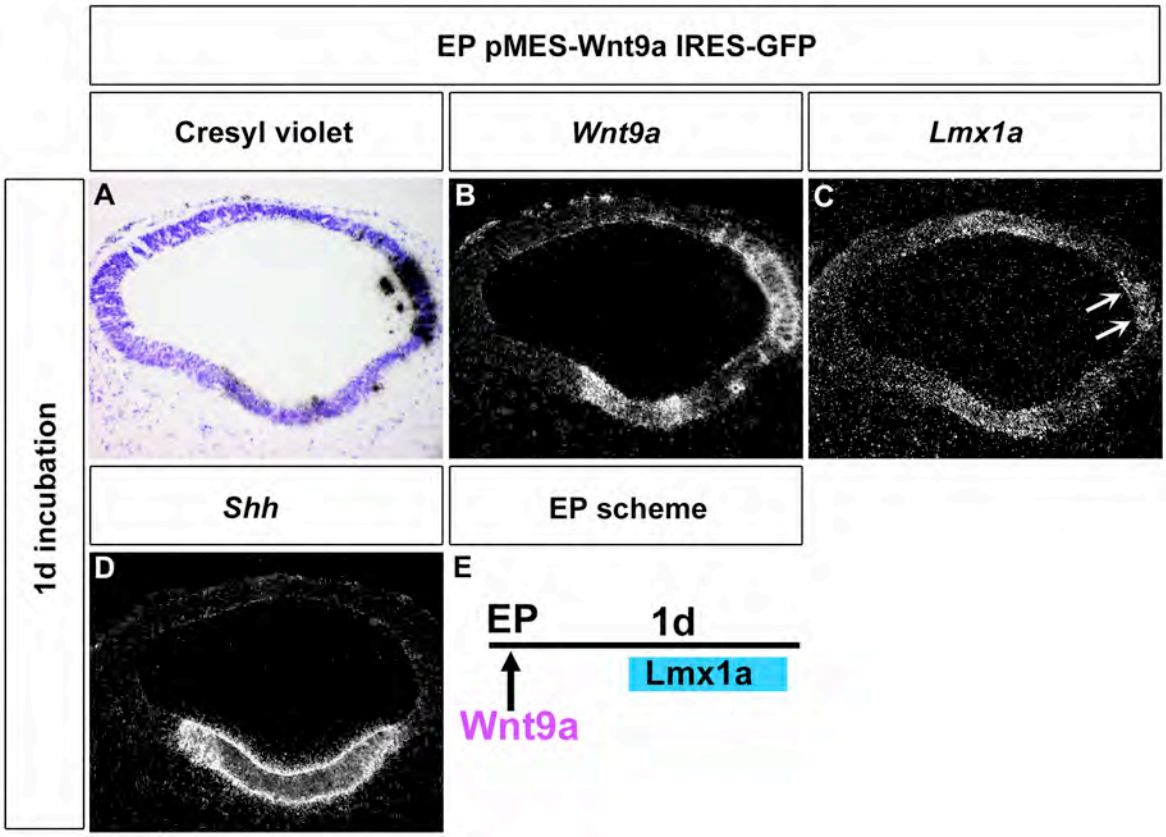

Figure 28: *Lmx1a* was induced ectopically 1d after electroporation of Wnt9a. (A-D) Consecutive coronal sections of a chicken brain 1d after Wnt9a electroporation. (A) Brightfield image of (B). The consecutive sections were hybridised with probes for *Wnt9a* (B), *Lmx1a* (C) and *Shh* (D). The arrows in (C) indicate the ectopic induction of *Lmx1a* after the Wnt9a overexpression. (E) Electroporation scheme: Wnt9a induces *Lmx1a* after 1d of incubation, but not *Pitx3, Nr4a2* or *Shh*.

3.2.2.2 Wnt9a overexpression induced *Pitx3* and *Shh* after 2d

Investigation of the mesDA marker genes after Wnt9a electroporation and an incubation period of 2 days revealed an ectopic induction of *Pitx3* (n=2/2; Fig. 29E). Surprisingly, not only induction of the mesDA marker gene was found after 2d of incubation, but also of *Shh* (n=3/3; Fig. 29C) and *Foxa2* (n=1/1; Fig. 29D). *Foxa2* is, like *Shh*, normally expressed in the floor and basal plate of the midbrain. For both genes it was shown in the

mouse that they are necessary for the development of mesDA neurons (Blaess et al., 2006; Ferri et al., 2007). However, *Shh* and *Foxa2* are activators and targets of each other (Sasaki et al., 1997), which means that the ectopic induction of one of these genes can be a secondary effect mediated by the other. Notably, the ectopic induction of *Pitx3, Shh* and *Foxa2* was found only in the basal plate and ventral alar plate of the midbrain (Fig. 29C, D, E; arrows), although *Wnt9a* was also ectopically expressed in the dorsal midbrain (Fig. 29B).

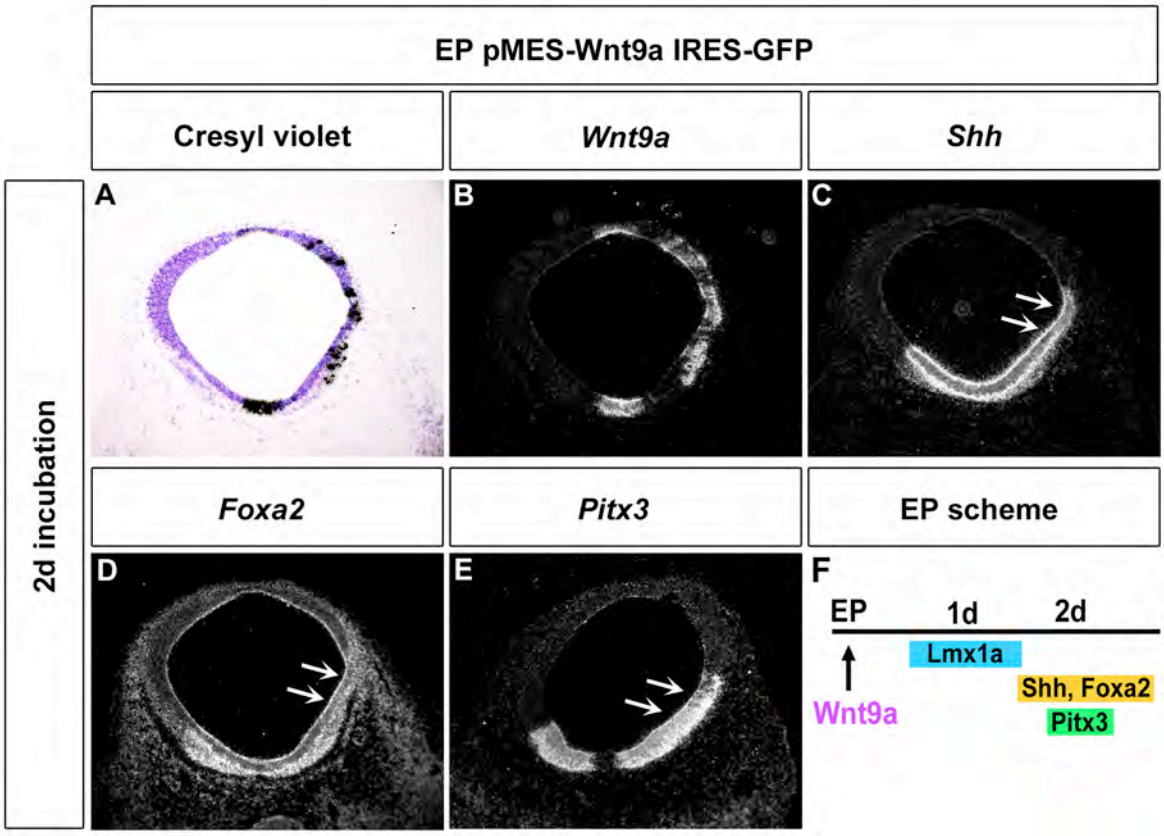

Figure 29: Ectopic Wnt9a is sufficient for the induction of *Pitx3*, *Shh* and *Foxa2* in the ventral midbrain after 2 days. (A-D) Consecutive coronal sections of chicken brains at E3.5 electroporated with Wnt9a. (A) Brightfield image of (B), hybridised with *Wnt9a*. The consecutive coronal sections were hybridised with probes for *Shh* (C) and *Foxa2* (D). (E) Coronal section of a chicken brain at E3.5, electroporated with Wnt9a and hybridised with a probe for *Pitx3*. Arrows indicate the ectopic induction of *Shh, Foxa2* and *Pitx3* in the ventral midbrain after Wnt9a electroporation. (F) Electroporation scheme: Wnt9a is sufficient to induce *Lmx1a* after 1d of incubation and *Shh, Foxa2* and *Pitx3* after 2d of incubation.

3.2.2.3 Electroporation of Wnt9a led to the induction of *Nr4a2* and *Ngn2* after 3d

After the electroporation of Wnt9a and an incubation of 3d, ectopic expression of the mesDA marker gene *Pitx3* was still clearly detectable (n=3/3; Fig. 30C, arrows). In addition, an ectopic induction of *Nr4a2* was detected now in the ventral midbrain (n=3/3; Fig. 30D, arrows). Again, the ectopic induction of these mesDA marker genes was restricted to the basal plate and ventral alar plate of the midbrain, although the overexpression of Wnt9a was also detected in the dorsal midbrain (Fig. 30B).

Recently, an involvement of the proneural factor *Neurogenin2* (*Ngn2*) has been demonstrated in the neurogenesis of mesDA neurons (Andersson et al., 2006; Kele et al., 2006). In the unelectroporated brain, the expression of *Ngn2* displays a gap at the alar-

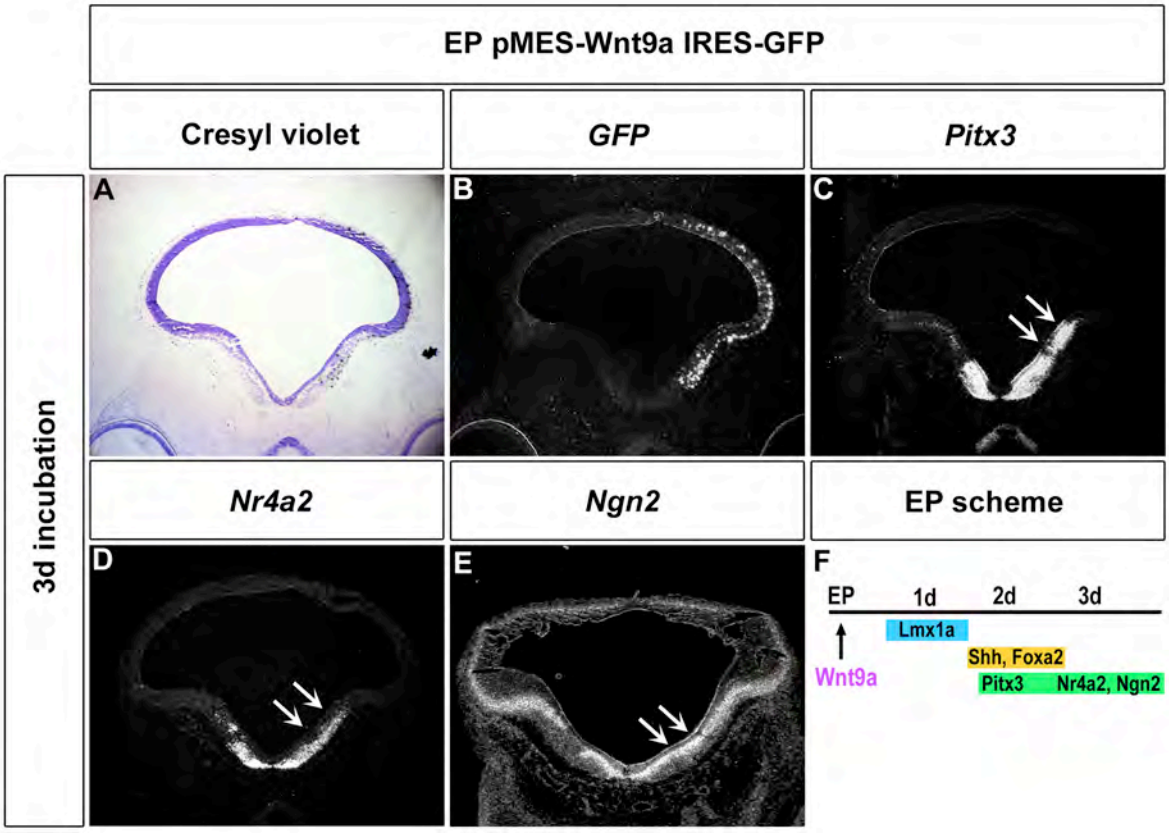

Figure 30: Electroporation of Wnt9a induced *Pitx3, Nr4a2* and *Ngn2*. (A-E) Coronal sections of a chicken brain at E4.5, electroporated with Wnt9a and incubated for 3d. (A) Brightfield image of (B). (B) Darkfield image of a coronal section hybridised with a probe for *GFP*. The *GFP* expression is equivalent to the EP site of Wnt9a. (C-E) Coronal sections of a Wnt9a electroporated chicken brain hybridised with probes for *Pitx3* (C), *Nr4a2* (D) and *Ngn2* (E). The arrows point to the ectopic expression domain in the ventral midbrain. (F) Electroporation scheme: EP of Wnt9a led to a transient induction of *Lmx1a* after 1d, ectopic induction of *Shh, Foxa2, Pitx3* after 2d and ectopic induction of *Nr4a2* and *Ngn2* after an incubation period of 3d.

basal boundary. Interestingly, this gap was not detected on the electroporated side, and *Ngn2* was expressed throughout the SVZ of the basal and ventral alar plate (n=1/2; Fig. 30D, arrow). In summary, overexpression of Wnt9a led to an induction of the mesDA marker gene *Pitx3* and of *Shh* after 2d and to an induction of *Nr4a2* and *Ngn2* after 3d.

As an ectopic induction of *Lmx1a* was detected 1d after Wnt9a electroporation, I asked whether ectopic *Lmx1a* expression was still detectable after 3d of incubation. Interestingly, no ectopic *Lmx1a* expression was visible after 3d of incubation (n=5/5; Fig. 31C). The study which described Lmx1a as an intrinsic determinant for mesDA development also identified the homeodomain protein Msx1 to be involved in the process of mesDA cell-fate specification (Andersson et al., 2006b). *Msx1* was described to be expressed in DA progenitors in the ventral midbrain at HH26 (E5) by Andersson et al. (2006b). Thus, I

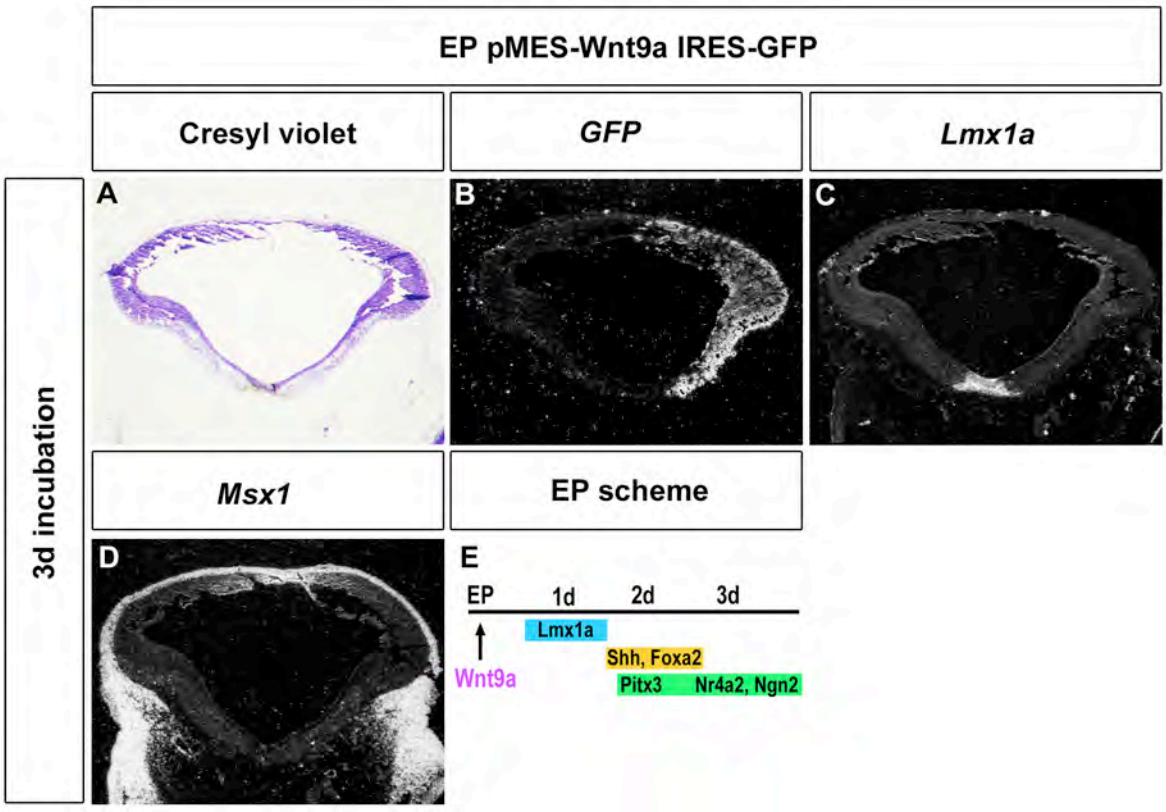

Figure 31: *Lmx1a* and *Msx1* are not induced 3 days after Wnt9a electroporation in the midbrain. (A-D) Coronal sections of a chicken brain at E4.5 after Wnt9a electroporation. (A) Brightfield image of (C) .(B-D) Darkfield images of consecutive coronal sections hybridised with probes for *GFP* (B), *Lmx1a* (C) and *Msx1* (D). (E) Electroporation scheme: Overexpression of Wnt9a led to a transient induction of *Lmx1a* after 1d, but *Lmx1a* expression was extinguished after 3d. Ectopic induction of *Shh, Foxa2, Pitx3* was detected after 2d and of *Nr4a2* and *Ngn2* after 3d.

investigated the expression of *Msx1* after Wnt9a electroporation as well. However, no ectopic induction of *Msx1* was detected in the ventral midbrain after 3d incubation (n=2/2; Fig. 31D).

3.2.2.4 Electroporation of Lmx1a induced ectopic expression of *Pitx3, Nr4a2* and *Shh* after 3d

The ectopic induction of *Lmx1a* after Wnt9a electroporation suggested that Wnt9a is acting upstream of Lmx1a. Next, it was examined whether Lmx1a can also induce *Pitx3* ectopically in the ventral midbrain of the chicken embryo as this has not been addressed by Andersson et al. (2006). Electroporation of Lmx1a resulted in a strong induction of *Pitx3* (n=8/8; Fig. 32B, arrows) and *Nr4a2* (n=4/4; Fig. 32C, arrows) in the ventral midbrain after 3d of incubation of the chicken embryo in line with Andersson et al. (2006b). Again, the ectopic induction of *Pitx3* and *Nr4a2* was restricted to the ventral part of the midbrain (basal plate and ventral alar plate). Due to the fact that Wnt9a induced *Lmx1a* after 1d of incubation and *Shh* after 2d, I next asked whether Lmx1a is also able to induce *Shh*. The ISH revealed a strong induction of *Shh* in the ventral part of the midbrain after Lmx1a electroporation and an incubation of 3 days (n=2/2; Fig. 32D, arrows).

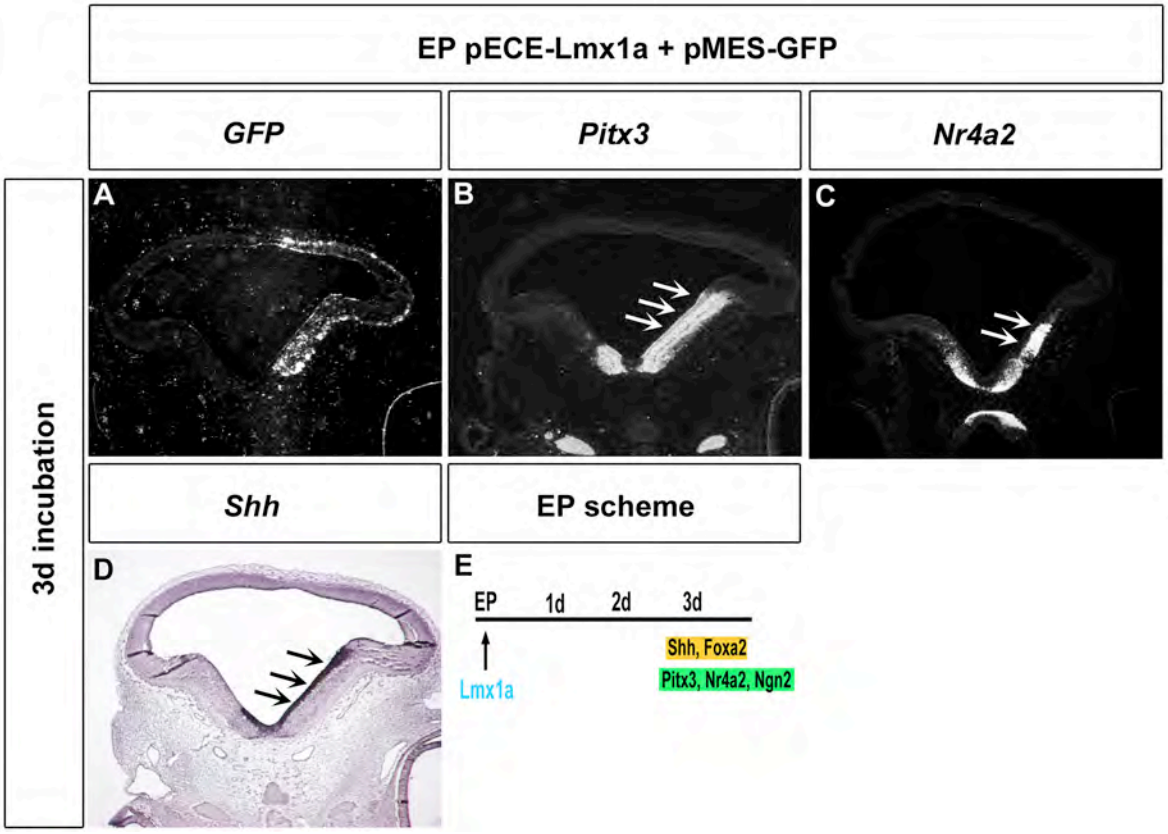

Figure 32: Electroporation of Lmx1a induced expression of *Pitx3, Nr4a2* and *Shh* in the ventral midbrain. (A-D) Coronal sections of a chicken brain at E4.5, electroporated with Lmx1a and incubated for 3 days. Darkfield images were taken from coronal sections hybridised with probes for *GFP* (A), *Pitx3* (B) and *Nr4a2* (C). (D) The brightfield image was taken from a section hybridised with a probe for *Shh*. The arrows indicate the ectopic induction *of Pitx3, Nr4a2* and *Shh* in the ventral midbrain after the Lmx1a overexpression. (E) Electroporation scheme: Electroporation of Lmx1a into the midbrain induces *Shh, Pitx3* and *Nr4a2* after 3d of incubation in the basal and ventral alar plate of the midbrain.

3.2.2.5 Overexpression of Lmx1a induced *Wnt9a* in the VZ after 3d

As only a transient induction of *Lmx1a* was found 1d after Wnt9a electroporation, which was not detectable after an incubation of 3 days, a mutual regulation could exist between these two genes. Therefore, Lmx1a was electroporated into the neural tube of chicken embryos. After an incubation time of 3 days, an ectopic induction of *Wnt9a* was found on the transfected side (n=5/8; Fig. 33C, arrows) in the same domain where *Pitx3* (n=8/8; Fig. 33D, arrows) and *Nr4a2* (n=4/4; Fig. 33E, arrows) were also induced ectopically. In addition, the proneural gene *Ngn2* was ectopically induced in the ventral diencephalon 3d after Lmx1a electroporation (n=6/6; data not shown). Notably, after 3d of incubation, the

ectopic induction of *Wnt9a* expression was restricted to the VZ, whereas ectopic *Pitx3* expression was found throughout the whole neuroepithelium (VZ, SVZ and MZ) and ectopic *Nr4a2* expression was detected only in the MZ.

Taken together, the overexpression of Lmx1a led to an induction of the mesDA marker genes *Pitx3* and *Nr4a2* as well as to an induction of *Wnt9a, Shh* and *Ngn2* after 3d of incubation. Noteably, the ectopic induction of all these genes was always restricted to the ventral part of the neural tube (basal and ventral alar plate).

To better characterize the genetic cascade, short-term incubation studies (1d and 2d) have to be done for the Lmx1a electroporation. Due to time-limitations of the PhD thesis these data are missing and the experiments still have to be performed.

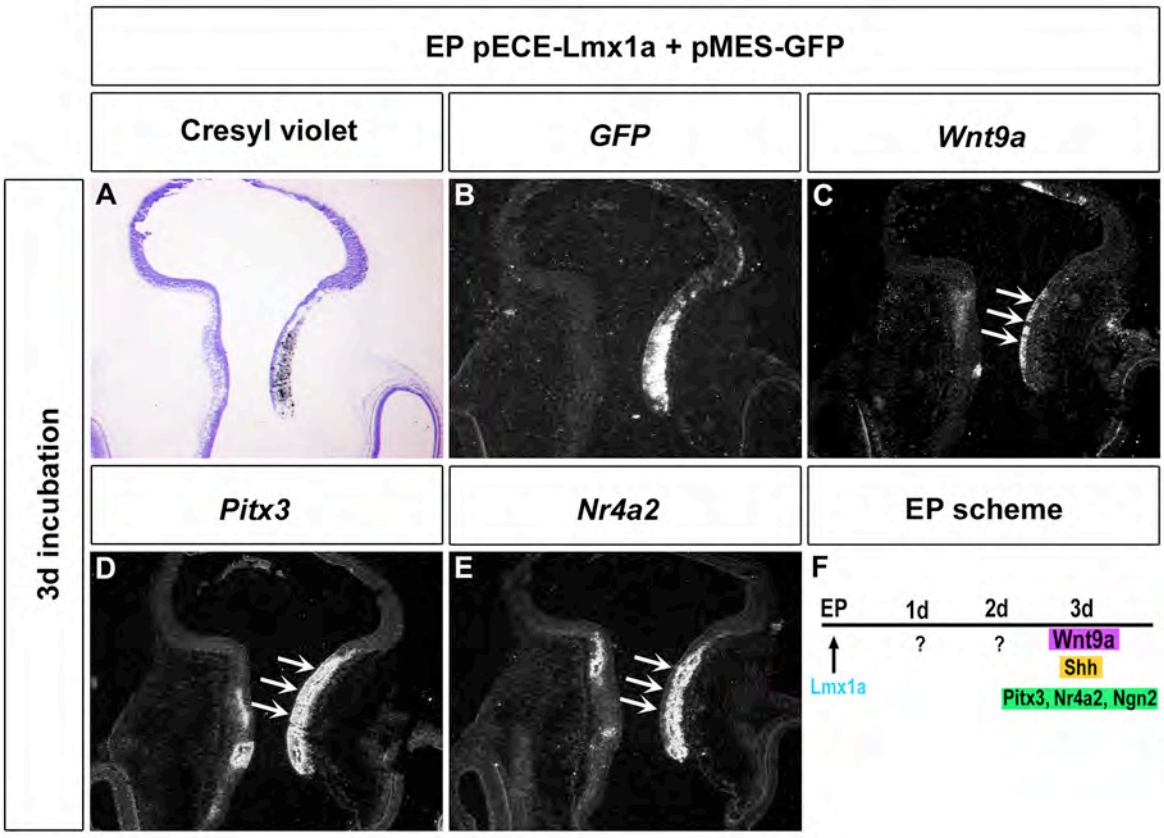

Figure 33: Ectopic induction of *Wnt9a* in the ventral diencephalon 3d after electroporation of Lmx1a. (A-E) Consecutive coronal sections of a chicken brain electroporated with Lmx1a. (A) Brightfield image of (B). The darkfield images show consecutive sections hybridized with probes for *GFP* (B), *Wnt9a* (C), *Pitx3* (D) and *Nr4a2* (E). (F) Electroporation scheme: EP of Lmx1a led to an induction of *Wnt9a, Shh, Ngn2* and the mesDA marker genes *Pitx3* and *Nr4a2* after 3d of incubation. Gene expression pattern after Lmx1a EP and 1d and 2d incubation are not known.

3.2.2.6 Shh acts downstream of Wnt9a and Lmx1a

Since I saw induction of *Shh* 2d after ectopic expression of Wnt9a (and 3d after overexpression of Lmx1a), the question arose whether *Shh* is downstream of Wnt9a/Lmx1a and upstream of *Pitx3, Nr4a2* and *Ngn2* expression. Furthermore, a previous study has shown that overexpression of Shh in the midbrain of chicken embryos led to an ectopic induction of mesDA neurons (Watanabe and Nakamura, 2000). Therefore, I investigated if an overexpression of Shh has an effect on the expression of *Wnt9a* and *Lmx1a* in the ventral midbrain. Interestingly, overexpression of Shh in the midbrain did not induce ectopically neither *Lmx1a* (n=5/5; Fig. 34C) nor *Wnt9a* (n=5/5; Fig. 34D) in the ventral midbrain after 3d of incubation. In addition, electroporation of an

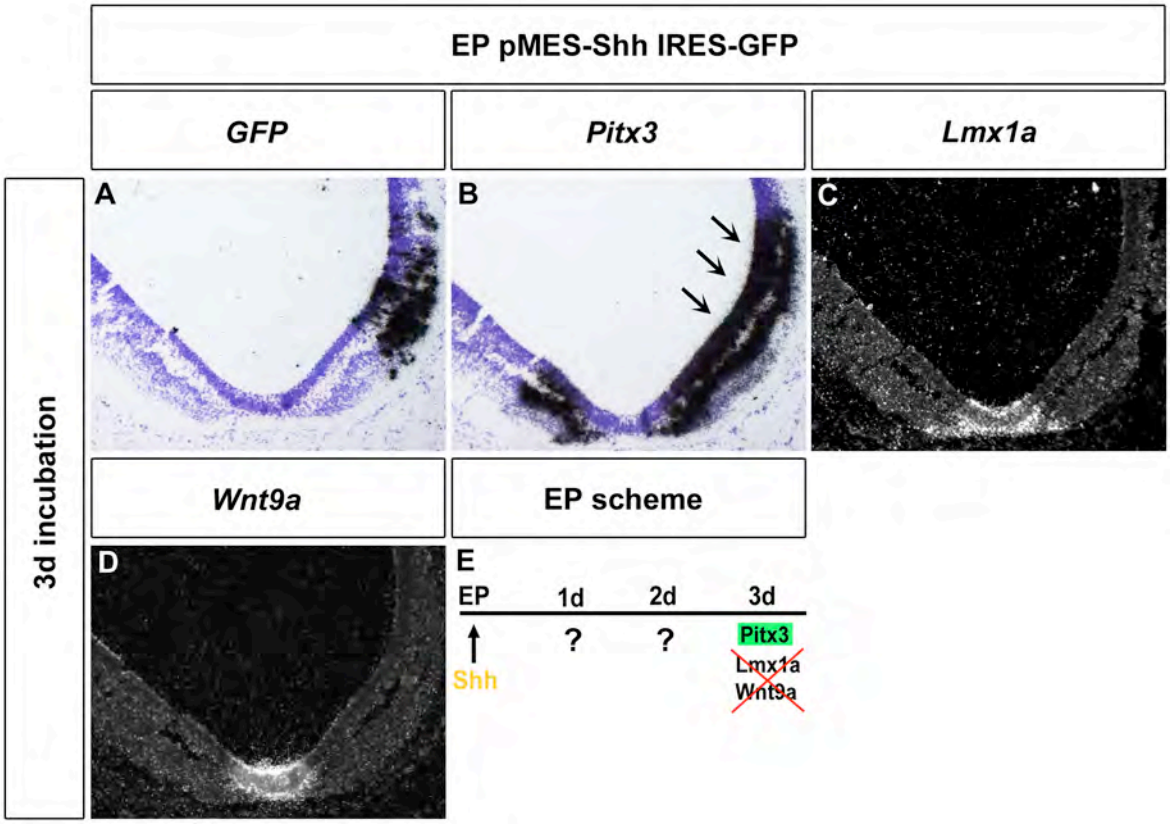

Figure 34: Overexpression of Shh in the midbrain induced *Pitx3*, but not *Lmx1a* or *Wnt9a* after 3 days. (A-D) Consecutive coronal sections of chicken embryo after electroporation with Shh and 3d of incubation. (A-B) Brightfield images taken from coronal sections hybridised with probes for *GFP* (A) and *Pitx3* (B). The arrows point at the ectopic *Pitx3* induction in the basal plate of the ventral midbrain. The darkfield images were taken from sections hybridised with probes for *Wnt9a* (D) and *Lmx1a* (C). (E) EP scheme: Electroporation of Shh into the midbrain induced *Pitx3*. *Lmx1a* and *Wnt9a* were not induced by ectopic Shh. The data for 1d and 2d incubation is still missing.

expression plasmid containing the chicken *Shh* cDNA induced ectopic *Pitx3* expression after 3d of incubation in the basal plate and ventral alar plate of the midbrain (n=3/3; Fig. 34B, arrows). Due to lack of time the data for 1d and 2d incubation is still missing. However, it will be very interesting to know whether *Wnt9a* or *Lmx1a* are transiently induced after Shh electroporation and a shortened incubation time.

Taken together, these results indicated that *Shh* is a downstream target of Wnt9a and Lmx1a in the chicken embryo since Wnt9a and Lmx1a are both able to induce *Shh* ectopically but not vice versa. The transient induction of *Lmx1a* by Wnt9a overexpression after 1d suggests a genetic cascade with Wnt9a inducing *Lmx1a*, which in turn induces *Shh* after 2d. Furthermore, the strong correlation of the expression patterns of *Shh* and *Pitx3* (see Fig. 13) and the temporal delay of the ectopic induction of *Pitx3* suggested that *Pitx3* is regulated primarily by the Shh signaling pathway in the chicken embryo and not by the Wnt9a pathway.

To substantiate this hypothesis, a bioinformatics analysis of the chicken *Pitx3* promoter (ENSGALG5613; version April 2006) was done for Gli protein binding sites. Gli-type zinc finger proteins (including Gli and Zic proteins) function as transcriptional mediators of the Hh signaling cascade and are implicated in the activation and repression of Hh target genes (reviewed in Koebernick and Pieler, 2002). The transcription factor Zic2 acts as a transcriptional regulator in Shh signaling (reviewed by J. Aruga, 2004). Interestingly, four Zic2 binding sides were found between the start codon (ATG) and 3000 bp upstream of the translation start in the *cPitx3* gene promoter (Fig. 35). Therefore, both electroporation and bioinformanitcs analyses strongly suggest that *Pitx3* is a Shh signaling target gene.

Figure 35: The Pitx3 promoter contains 4 Zic2 binding sites. The 5´UTR and promoter region of the *Pitx3* gene is shown up to 3kb. Four Zic2 binding sites could be found at positions -1110bp, -1878bp, -2943bp and -2980bp upstream of the translation start (ATG).

To address the question whether Wnt9a indeed acts upstream of Shh, I decided to overexpress Wnt9a and simultaneously block Shh signaling using cyclopamine, a potent antagonist of Shh signaling (Incardona et al., 1998). If the inhibition of Shh signaling by cyclopamine in the presence of Wnt9a leads to a failure to induce *Pitx3,* it is very likely that the induction of this mesDA marker gene by Wnt9a is mediated by Shh. To determine that Shh signaling was inhibited by cyclopamine, the expression of the Shh receptor *Patched1* was used as a control, because *Patched1* is a direct Shh target (Ingham et al., 1991). However, no effect was detected between the *Patched1* transcription in cyclopamine treated embryos (n=3/3) and control (HBC treated; n=3/3) embryos (Fig. 36A

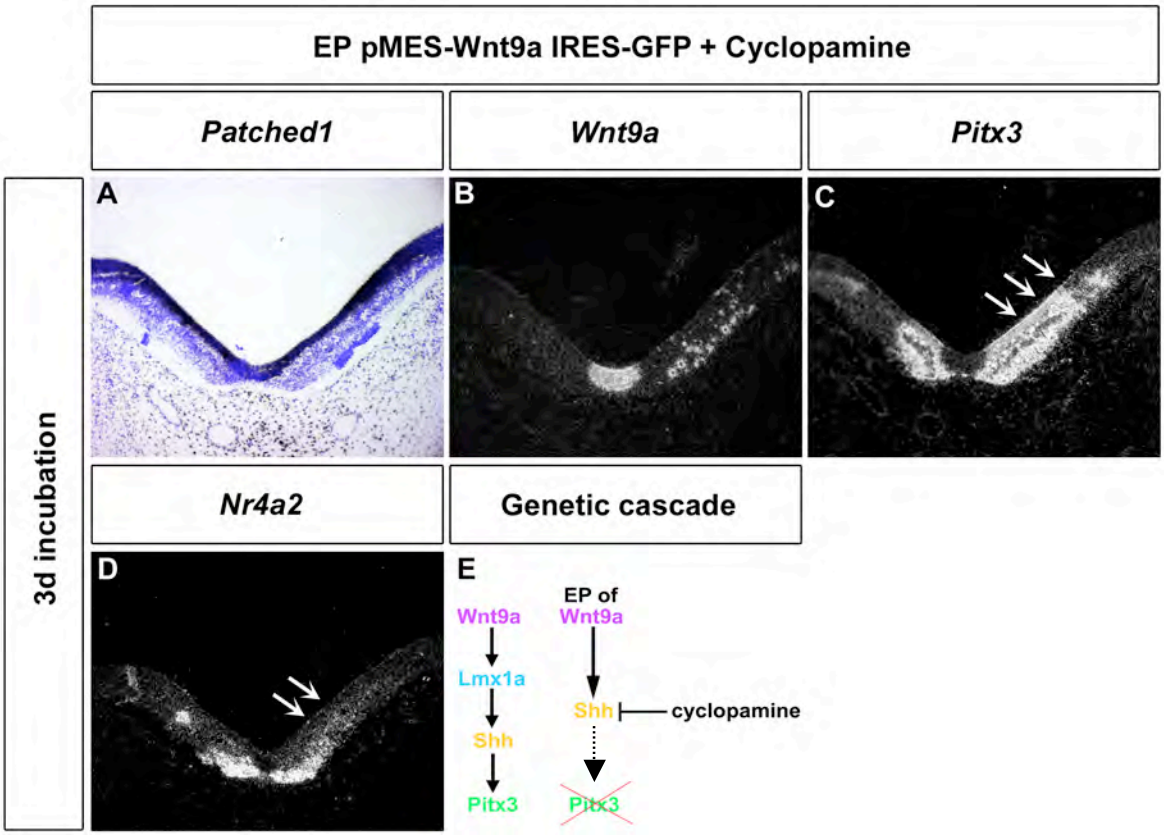

Figure 36: Pilot experiment for blocking Shh signaling by cyclopamine in the presence of Wnt9a. (A-D) Consecutive coronal sections of a chicken embryo at E4.5, electroporated with Wnt9a and simultaneously treated with cyclopamine. (A) Brightfield image taken from a section hybridised with a probe for *Patched1*. The darkfield images show the corresponding sections hybridised with probes for *Wnt9a* (B), *Pitx3* (C) and *Nr4a2* (D). (E) Postulated hypothesis of the genetic cascade (left side): Wnt9a transiently induces *Lmx1a*, which in turn induces *Shh*, which activates *Pitx3* transcription. Experimental approach to test hypothesis (right side): electroporation of Wnt9a and simultaneously blocking of Shh signaling by cyclopamine should lead to a failure in the induction of *Pitx3* expression if no alternative pathway is required.

and data not shown) after electroporation of Wnt9a and an incubation of 3d. Consequently, the ectopic induction of *Pitx3* (Fig. 36C, arrows) and *Nr4a2* (Fig. 36D, arrows) was detected 3d after Wnt9a overexpression (Fig. 36B). I concluded that the inhibition of Shh signaling by cyclopamine was not successful with this experimental set-up. Therefore, the electroporation of Wnt9a simultaneous cyclopamine treatment still have to be optimized and repeated.

4 Discussion

In this work I showed a first detailed analysis of the expression patterns for the chicken orthologues of the mouse mesDA marker genes *Aldh1a1*, *Nr4a2*, *Pitx3*, *Lmx1b* and *Th* during early chicken embryonic development. The analysis revealed striking differences between chicken and mouse in the spatiotemporal expression for some of the chicken orthologues. Expression of *Nr4a2* and *Pitx3* is found first in a diencephalic domain, whereas *Aldh1a1* is not expressed in the anterior neural tube of the chicken embryo. In addition, in the chicken embryo *Pitx3* expression is detected at least 1,5d earlier than in the mouse embryo. This early diencephalic expression of *Pitx3* was confined to proliferating cells. The knowledge of the expression of mesDA marker genes enabled the functional study of the Wnt-pathway in the development of mesDA neurons in the chicken embryo. Overexpression of Wnt9a led to the ectopic induction of the mesDA marker genes *Pitx3* and *Nr4a2* in the ventral midbrain. In addition, *Shh* was identified as a downstream target of Wnt9a and the mesDA marker gene *Pitx3* as a very likely transcriptional target of Shh in the chicken embryo.

4.1 Similarities and differences in the development of mesDA neurons between chicken and mouse embryos

I found striking differences between chicken and mouse in the spatiotemporal expression patterns for some of the analyzed genes, which may reflect the evolutionary differences of the mesDA system between birds and mammals. Although I do not provide conclusive evidence that the *Th*- expressing cells in this study are truly mesDA neurons due to the lack of immunohistochemical and functional data, I do consider that the cells located in the chicken midbrain at E6.5 are indeed the mesDA neurons because of the following reasons: 1) Although their suggested progenitors arise in the ventral diencephalon corresponding to p1/2, the cells expressing *Th* mRNA are located in the caudal part of the ventral midbrain and are arranged in a shape reminiscent of the mouse SN and VTA, in agreement with Th-immunohistochemical data from the developing chicken embryo (Puelles and Medina, 1994); 2) These cells additionally express all or most of the marker genes that have been

described in the mouse as necessary for the development of these mesDA neuronal population.

The expression of the *Nr4a2*, *En1* and *Lmx1b* genes in the chicken embryo had a similar spatiotemporal pattern as in the mouse embryo. *Nr4a2* transcription was first detected in the E3.5 chicken embryo (mouse E10.5) in what appeared to be the postmitotic progeny of the *Pitx3*-expressing cells. This is in good agreement with the mouse data (Wallen et al., 1999; Zetterstrom et al., 1997). Similar to what has been noted for the early diencephalic detection of *Pitx3* mRNA in the chicken embryo, expression of *Nr4a2* initiated in a more rostral domain than in the mouse embryo. At later stages (E6.5; mouse E13.5), *Nr4a2* expression almost completely overlapped with the *Th*-positive cells in the ventral midbrain of the chicken embryo. The chicken Nr4a2 nuclear receptor may therefore have a similar function as in the mouse, namely to initiate transcription of the *Th* gene in mesDA precursors (Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Zetterstrom et al., 1997). The expression domain of *En1* was initially broad in the caudal midbrain and rostral hindbrain and narrowed down at later stages, until it became centered at the MHB in the E5.5 chicken embryo (mouse E12.5) (Shamim et al., 1999). Overlapping expression domains of *En1* and the DA marker genes were detected in the ventral midbrain of E5.5 and E6.5 chicken embryos, suggesting that chicken En1 may play a similar role in mesDA neuron maintenance as in the mouse (Alberi et al., 2004; Simon et al., 2001). Transcription of the *Lmx1b* gene is initiated in the developing chicken embryo even before neurulation starts and later encompasses a broad domain of the neural tube similar to the mouse embryo (Adams et al., 2000; Asbreuk et al., 2002; Matsunaga et al., 2002; Smidt et al., 2000). Expression of this gene was confined to the ventral and dorsal midline of the neural tube at early stages of chicken development (E2.5), overlapping with the *Pitx3*-positive DA progenitor domain. Later on, expression of *Lmx1b* was more restricted in the midbrain tegmentum and appeared to be confined to the *Pitx3*-positive progenitor cells and postmitotic *Th*-expressing mesDA neurons. This pattern resembles the later expression of *Lmx1b* in the mouse embryo (Asbreuk et al., 2002). One report has suggested a functional requirement of the mouse Lmx1b protein in mesDA neuron development (Smidt et al., 2000). Whether this is also true for the chicken embryo remains to be established.

For the differences in the expression pattern of the mesDA marker genes, three important points can be made between the chicken and mouse embryo. First, the *Pitx3* gene was the earliest marker to be expressed in the ventral neural tube of the chicken embryo. Its expression was detected already at E2.5 of chicken embryonic development, which is equivalent to mouse E10, and thus started at least 1.5 days earlier than in the mouse embryo (Smidt et al., 2004b; Smidt et al., 1997). Furthermore, expression of *Pitx3* in the chicken embryo was first confined to a region corresponding to p1/2 of the ventral diencephalon and was initially absent from the midbrain territory. Such a rostral expression domain was not described before for the *Pitx3* gene in the mouse embryo, although the early anterior ventro-lateral mPitx3-expressing cells reported by Maxwell et al. (2005) may also be reaching into a diencephalic territory. At stage E3.5 in the chicken embryo, the *Pitx3* mRNA was expressed by both proliferating DA progenitors located in the ventricular zone and their offspring, located in the outer (marginal) layer of the diencephalic neural tube. The data from the mouse embryo analysis have excluded an expression of *Pitx3* in proliferating cells and suggested that *Pitx3* transcription is initiated in postmitotic cells shortly before or after they begin to express *Th* (Maxwell et al., 2005; Smidt et al., 2004a; Smidt et al., 1997; van den Munckhof et al., 2003). Therefore, the temporal expression of the mouse *Pitx3* gene is restricted to later time-points and to postmitotic mesDA precursors, indicating that Pitx3 may have lost some of its earlier functions in proliferating progenitors and/or acquired new ones in their progeny at the transition from birds to mammals. However, taking a closer look at later stages of embryonic mouse development (E14), I discovered a ventral diencephalic expression domain of *mPitx3*. These cells appear to be dopaminergic neurons as confirmed by an *in situ* hybridization for *Th* on consecutive sections. My findings revealed that the expression of *mPitx3* in the developing mouse embryo is not restricted exclusively to the ventral midbrain as described before (Smidt et al., 1997; Zhao et al., 2004), but is also detected in a ventral diencephalic domain at later stages comparable to the chicken embryo.

Second, expression of the *Th* gene was first detected at E6.5 in the chicken embryo, equivalent to mouse E13.5, and thus four days after *Pitx3* expression had initiated and two days later than in the developing mouse embryo (Wallen et al., 1999; reviewed by Prakash and Wurst, 2006). It should be noted that Puelles and Medina (1994) reported the first appearance of Th- and DA-immunoreactive cells in the midbrain tegmentum of the chicken

embryo at E8, i.e. 1.5 to 2 days after the first detection of the *Th* mRNA in the same region as described in the present study. The reason for this time lag in mRNA expression and protein detection is unclear at present, but may be comparable to the mouse embryo. The expression domain of *Th* mRNA in the developing mouse embryo is much broader and appears earlier than the corresponding domain of Th protein (Marín et al., 2005). In addition, the expression site of *Th* comprised only a subpopulation of the *Pitx3*-positive cells in the ventral midbrain of the E6.5 chicken embryo. The *Th*- and *Pitx3*-positive neurons were confined to a dorso-lateral domain of the posterior ventral midbrain, whereas more medially located cells at the same level were only expressing *Pitx3*. This pattern may reflect a similar initial segregation of the Th and Pitx3 domains in the mouse embryo at E12.5, although it should be noted that the *Pitx3*-positive domain in the chicken embryo was in general much broader than in the mouse embryo (Maxwell et al., 2005). Assuming that at E4 the midbrain epithelium of the chicken embryo is predominantly composed of undifferentiated precursors (Bayly et al., 2007) and that *Pitx3* is expressed in proliferating cells before detection of *Th* expression, I therefore concluded that *Pitx3* can be used as a marker for both mesDA precursors and postmitotic neurons in the chicken embryo.

Third, *Aldh1a* genes are not expressed in the developing chicken midbrain, confirming previous data obtained by WISH and IHC studies (Berggren et al., 1999; Blentic et al., 2003; Suzuki et al., 2000; Swindell et al., 1999). By using the more sensitive radioactive ISH method, I was unable to detect other *Aldh1a* expression sites than the published ones except for *Aldh1a2*. The *Aldh1a1* gene was expressed in the dorsal neural retina of the E3.5 chicken embryoin accordance with Blentic et al. (2003) and Suzuki et al. (2000). This expression domain has also been described in the mouse embryo (Fan et al., 2003; Niederreither et al., 2002). The rather strong *Aldh1a2* expression in the rostral head mesenchyme at E3.5 has not been reported so far, but this may have been masked in previous studies by the even stronger *Aldh1a2* expression in more caudal regions of the chicken embryo (Berggren et al., 1999; Blentic et al., 2003; Swindell et al., 1999; Tsukui et al., 1999). The *Aldh1a3* mRNA was found to be strongly expressed in Rathke's pouch (ventral forebrain) on midsagittal sections and in a ring encompassing the isthmic constriction, in agreement with previous data (Blentic et al., 2003; Suzuki et al., 2000). Thus, the specific expression of Aldh1a1 in mesDA progenitors of the developing mouse embryo (Wallen et al., 1999) may be an acquisition of the mammalian lineage, because

another avian species also lacks *Aldh1a1* expression in the ventral midbrain (Reijntjes et al., 2005). Since a functional implication of this retinoic acid (RA) synthesizing enzyme in mesDA neuron development has not yet been proven in the mouse, the relevance of this finding remains unclear at present.

Although I found differences between the chicken and the mouse, I do regard the cells located in the ventral midbrain of the chicken embryo as the true mesDA neurons, as they do express most of the marker genes that have been described in the mouse as required for mesDA neuron development. The differences could represent evolutionary attributes which have evolved during the transition from anamniotes to amniotes.

4.2 The ontogeny of chicken mesDA neurons may recapitulate part of their phylogeny

A striking result of this study was the initially very rostral (diencephalic) location of the *Pitx3* and *Nr4a2* expression domains in the E2.5/E3.5 chicken embryo. Later in development, these domains were detected in progressively more posterior regions of the ventral midbrain. Whether this caudal progression reflects a migration of the corresponding cells or a successively more caudal induction of the corresponding genes was investigated by two different experimental approaches. A real evidence for a caudal migration of mesDA precursors came from the GFP electroporation experiment. GFP-labeled cells were found in the ventral midbrain after electroporation of an NLS-GFP construct into the ventral diencephalon. Since migrating GFP-positive cells were found between the electroporation site and the midbrain only after 1d but not after 4d, it can be concluded that the migration of the cells was an early event in this time period of 3d. The explant cultures rather suggest that an inductive signal from the diencephalon, e.g. from the secondary organizer ZLI (Kiecker and Lumsden, 2004) has to be present, as they were dissected at a time when no *Pitx3* expression was detected in the ventral midbrain. The mesDA marker gene *Pitx3* was not induced in cultured isolated midbrain explants, whereas it was induced/expressed in whole brain and forebrain. Additionally or alternatively, attractive guidance cues emanating from the MHB or a physical intactness of a migratory pathway between the di- and mesencephalon may be required *in vivo*. It has already been shown that the transcription factor En2, which is expressed at the MHB, can act as a guidance

molecule (Brunet et al., 2005). In conclusion, there is evidence that at least some of the mesDA neurons have a diencephalic origin in the chicken embryo.

The mesDA system appears to be a more recent acquisition during vertebrate evolution (reviewed by (Smeets and Gonzalez, 2000; Vernier et al., 2004). Neither ancestral chordates such as *Ciona intestinalis* and *Branchiostoma lanceolatum* nor lower vertebrates (anamniotes) such as bony fish possess a mesDA population (Moret et al., 2005; Moret et al., 2004; Rink and Wullimann, 2002a; Smeets and Reiner, 1994; Smeets and Gonzalez, 2000). In these species, DA neurons are located only in the telencephalic and diencephalic region of their brains. Based on their projection areas and functions, however, it was suggested that some forebrain DA subpopulations are homologous to the mesDA system found in higher vertebrates (amniotes) such as reptiles, birds and mammals (Marin et al., 1998; Rink and Wullimann, 2002b; Smeets and Gonzalez, 2000; Vernier et al., 2004). The mesDA system may thus have emerged during evolution at the transition from anamniotes to amniotes from a subpopulation of diencephalic DA neurons. It was indeed suggested that a subpopulation of the mesDA system, namely the most rostral part of the A9 (SN)/A10 (VTA) groups, develops earlier than the rest of the mesDA neurons in all higher vertebrate species studied so far (Marin et al., 1998). Furthermore, this rostral A9/A10 subpopulation arises within the diencephalic prosomeres (p1/2), where it remains even in adulthood. The majority of the SN and VTA mesDA neurons in mammals emerge only later in development by a caudal expansion and probably lateral migration of the rostral mesDA subpopulation (Marin et al., 1998). The mesDA neuronal population may therefore recapitulate its phylogenetic origin during its early ontogeny in all higher vertebrates. In agreement with this hypothesis, the early appearance of *Pitx3*-positive proliferating mesDA progenitors in a diencephalic territory of the chicken embryo may correlate with a recent report about the initial expression of the mouse *Th* mRNA (but not protein) in a diencephalic region of the mouse embryo (Marin et al., 2005). Nevertheless, I found striking differences in the development of the mesDA system between chicken and mouse. These differences may therefore reflect the evolutionary distance between the sauropsids (reptiles and birds) and mammals, which have emerged from a common ancestor in the vertebrate lineage.

However, the pre-requisite for drawing developmental similarities between the chicken and mouse mesDA system is the knowledge of functional similarities for the key factors

regulating this process. Therefore, functional studies for the mesDA marker gene *Pitx3* were performed in the chicken embryo. Since the experiments to elucidate the functional role of Pitx3 during early chicken development did not give the expected results, I asked whether this was due to functionality or efficiency of the morpholinos/expression constructs. My best explanation is that the morpholinos and the activator/repressor constructs were not as efficient as expected, since at least the functionality of the constructs was confirmed in the zebrafish system before using them for the *in ovo* electroporation. One reason for this inefficiency could be that the rapid growth of the midbrain tissue during early chicken development diluted the concentration of the morpholino/construct DNA in the tissue beyond effectiveness. Gene dosage is important for all mesDA marker genes in the mouse, as heterozygous mutants for all known murine mesDA genes do not show a developmental phenotype and, in particular, mice heterozygous for the *Pitx3* gene also show no defects in the mesDA system (Zhao et al., 2004; Maxwell et al., 2005). It is therefore feasible that a diluted concentration of the morpholinos/constructs was insufficient to disturb the normal generation of mesDA neurons in the chicken embryo. An additional explanation could be the short time period in which the morpholinos/constructs were effective. With the conventional technique of electroporation, the expression of a transgene cloned into an expression vector is only transient, lasting merely two to three days even though a ubiquitous promoter is driving the transgene (Sato et al., 2007). The period of action of the morpholinos is also limited to a few days (Ekker and Larson, 2001). Therefore, the long incubation time of 6 days could have led to a recovery of the mesDA system, if the morpholinos/constructs lost their activity after two to three days. However, it also cannot be excluded that the chicken *Pitx3* gene has only a function for the survival and/or maintenance of mesDA neurons. Recently, Filippi and colleges (2007) analysed the function of *pitx3* in the development of DA neurons in the zebrafish. The authors showed that *pitx3* is expressed in both proliferating and early differentiating progenitor cells of the posterior tuberculum, a diencephalic DA domain in the zebrafish, while *nr4a2* is expressed in postmitotic differentiating neurons in the same area, similar to the expression pattern of these two genes in the chicken embryo. The morpholino knock-down of *pitx3* did not affect the specification or differentiation of DA neurons in the zebrafish. However, a double-labeling for *pitx3* and Th revealed that these two genes are not co-expressed in the zebrafish, which is in contrast to mouse and

chicken embryos.

The comparison of mesDA marker gene function between two or more vertebrate species will help to establish the phylogenetically conserved pathways in mesDA neuron development. To address the question what the exact roles of Pitx3, Nr4a2 and Lmx1b are in the development of mesDA neurons in the chicken embryo, gain- and loss-of-function studies have to be done. A recently developed transposon-mediated gene transfer in chicken embryos (Sato et al., 2007) can be used to circumvent the efficiency problem because of the too strong dilution and the too short active period of the morpholinos/constructs. With this novel technique, the transgene is stably integrated into the genome, which results in persistent expression for at least 10 days (Sato et al., 2007).

4.3 The functional analysis of chicken *Wnt9a* in the ventral midbrain revealed conserved and new mechanisms in mesDA neuron development

In the developing CNS, Wnt signaling has been shown to control proliferation, differentiation, cell survival and neural connectivity (Wodarz and Nusse, 1998; Ciani and Salinas, 2005). A recent publication demonstrated that Wnt1 plays a crucial role in the generation of mesDA neurons in the mouse embryo (Prakash et al., 2006). In that study, it has been shown that Wnt1 exerts two different functions in the development of mesDA neurons. Early in this process, Wnt1 is involved in the establishment of the mesDA progenitor domain by regulating a genetic network in the ventral midbrain. Later in development, Wnt1 is required for the terminal differentiation of the mesDA neurons, probably by regulating the transcription of *Pitx3*, since no Pitx3-expressing cells are generated in the absence of Wnt1 (Prakash et al., 2006). I therefore was interested in establishing whether Wnt1 or another Wnt exerts a similar function in mesDA neuron development in the chicken embryo.

Analysis of the expression patterns of some of the *Wnt* genes in the chicken embryo revealed a partly conserved expression of the mouse and chicken orthologues in some cases (McMahon et al., 1992; Parr et al., 1993). However, striking differences were found between the *Wnt1* and *Wnt9a* expression domains in the mouse and chicken embryo. While *cWnt1* was not expressed in the ventral cephalic flexure of the chicken embryo the *cWnt9a*

gene was strongly expressed there. In addition, the expression pattern of *Wnt9a* during early chicken development, with an early expression throughout the whole neuroepithelium and a later restriction to the SVZ and VZ of the cephalic flexure, strongly resembles the murine *Wnt1* expression. In the mouse embryo the expression of *Wnt1* comprises the whole neuroepithelium at E10.5 (chicken E3) and becomes restricted to the VZ at E12.5 (chicken E5)(Fischer, 2006). It therefore appeared that cWnt9a may be the functional homologue of mouse Wnt1. Since the only published expression data for the murine *Wnt9a* gene were for the early blastula (Kemp et al., 2005) and in the forming synovial joints of the developing mouse limb (Guo et al., 2004), I assessed Wnt9a expression in the developing mouse embryo. The murine *Wnt9a* gene also has a ventral expression domain in the diencephalon at E9.5, but the expression level is very weak at this stage and is subsequently lost during development. Thus, the expression domains of the *Wnt1* and *Wnt9a* genes in the cephalic flexure were not conserved between the mouse and chicken, and either gene may have substituted for the other. Since Wnt9a has been described to act through the canonical signaling pathway (Guo et al., 2004) as it is also true for murine Wnt1 (Castelo-Branco et al., 2004), cWnt9a seems to be a good candidate for having taken over the function of Wnt1 in the chicken embryo. Therefore, my functional electroporation studies focused on the *Wnt9a* gene and the question whether this factor has substituted the function of Wnt1 for mesDA neuron development in the chicken embryo.

The overexpression of Wnt9a in the chicken embryo led to an ectopic induction of the mesDA marker genes *Pitx3* and *Nr4a2* in the ventral part of the midbrain. This is in line with the recent report from Prakash et al. (2006), where ectopic *Wnt1* expression induced *Pitx3* and *Nr4a2* in the ventral hindbrain of transgenic mice. In addition, an ectopic induction of the proneural gene *Ngn2* was observed after electroporation of Wnt9a. This is consistent with recent findings that Ngn2 is also required for the development of mesDA neurons in mice, as a loss of this gene specifically affects the neurogenesis of mesDA neurons (Kele et al., 2006; Andersson et al., 2006a). The ectopic induction of all three genes, *Pitx3, Nr4a2* and *Ngn2,* after Wnt9a electroporation in the chicken embryo indicates that Wnt9a is sufficient to induce mesDA neuron markers and probably their specification. However, ectopic induction of the *Th* gene, which would indicate terminally differentiated mesDA neurons capable of synthesizing DA, was not detected after an incubation time of 5-6 days. Importantly, the ectopic induction of the mesDA marker genes was only

detectable after an incubation period of 2d, which means that there is a delay of 2 days also for the ectopic *Th* induction. Thus, a longer incubation time of seven or more days would be required to detect an ectopic induction of *Th*. However, survival rate of electroporated chicken embryos is strongly reduced with longer incubation times. As the transient expression of the transgene vanishes after 3 days, it is also possible that this time window was not enough to firmly turn on the genetic cascade necessary for the generation of terminally differentiated mesDA neurons. Furthermore, the delayed induction of *Pitx3, Nr4a2* and *Ngn2* indicates that Wnt9a does not act directly, but that the ectopic induction of these mesDA marker genes is very likely secondary to the action of other factors.

Recently, Andersson et al. (2006b) identified the homeodomain transcription factors Lmx1a and Msx1 as involved in the generation of mesDA neurons in mouse and chicken embryos. The authors showed by *in ovo* electroporation experiments that Lmx1a is sufficient and required for the induction of mesDA neurons in chicken embryos (Andersson et al., 2006b). However, analysis of the *dreher* mouse mutant, which carries a mutation in the murine *Lmx1a* gene, indicated the existence of redundant/additional pathways, as still 70% of the mesDA neurons developed normally in *dreher* mutants (Ono et al., 2007). Previous studies have already suggested that two independent pathways exist during the terminal differentiation of mesDA neurons: a Nr4a2-controlled pathway required for the initiation of *Th* transcription (Kim et al., 2003; Saucedo-Cardenas et al., 1998; Smits et al., 2003; Zetterstrom et al., 1997), and an Lmx1b/Wnt-controlled pathway necessary for *Pitx3* expression (Prakash et al., 2006; Smidt et al., 2000). As a knock-down of Lmx1a via RNAi in chicken embryos led to the loss of Nr4a2 (Andersson et al., 2006b), it could be considered that Lmx1a is controlling the Nr4a2- pathway. The identification of a possible link between the Wnt- and Lmx1a- pathways was one goal of the electroporation studies. Notably, the overexpression of Wnt9a led only to a transient induction of *Lmx1a*. Therefore, an explanation for the delay of 2d in the ectopic induction of the mesDA marker genes *Pitx3* and *Nr4a2* in the chicken embryo after Wnt9a electroporation would be that this was mediated by the transient expression of *Lmx1a*. In line with this result is the observation that *Lmx1a* is ectopically induced in the floor plate of the rostral hindbrain in the $En1^{+/Wnt1}$ mouse mutant even before *Nr4a2* by ectopic *Wnt1* (unpublished results).

I therefore analyzed the gene expression patterns/genetic cascade downstream of Lmx1a after ectopic expression of this factor in the chicken midbrain. Lmx1a was able to induce

expression of *Pitx3* and *Nr4a2* after 3d, as partly reported by Andersson et al. (2006b). Notably, however, an ectopic induction of *Wnt9a* was detectable in the SVZ and VZ of the rostral neural tube after Lmx1a overexpression. Recent studies have indeed shown the capability of LIM- homeodomain proteins to induce *Wnt* expression. Lmx1a was able to induce *Wnt1* in the dorsal aspect of the spinal cord, thus expanding the roof plate (Chizhikov and Millen, 2004), whereas Lmx1b was able to induce *Wnt1* in the dorsal midbrain of chicken embryos (Adams et al., 2000; Matsunaga et al., 2002) or in zebrafish embryos (O´Hara et al., 2005). These results suggest that a mutual feedback mechanism exists between Wnt9a and Lmx1a leading to the induction of mesDA neurons. Noteworthy, the ectopic induction of Lmx1a after Wnt9a electroporation did not correlate with a simultaneous induction of the mesDA marker genes Pitx3 and Nr4a2. It is therefore likely that yet another factor acts downstream of Lmx1a.

Indeed Wnt9a was able to induce ectopic *Shh* expression in the chicken midbrain, suggesting a link between the Wnt- and the Hh- pathway in the generation of mesDA neurons. Several studies have already shown an interaction of these two pathways. During somite formation, Gli2 and Gli3 are subject to positive and negative regulation, respectively, by canonical Wnt signaling from the surface ectoderm and neural tube (Borycki et al., 2000). Interestingly, a recent study showed that Wnt9a temporally and spatially regulates expression of Indian Hedgehog, another member of the Hedgehog (Hh) family, through the canonical pathway during chondrogenesis in the mouse embryo (Später et al., 2006). Furthermore, ectopic expression of Shh in the chicken midbrain led to the induction of Pitx3 after 3d. Although intermediate time points are still missing to dissect the genetic cascade downstream of Shh, it appears that Shh is mediating the Wnt9a and Lmx1a signal responsible for the generation of mesDA neurons in the chicken embryo. Although this contrasts with the study by Prakash et al. (2006), where no correlation between the Shh expression and the absence or presence of mesDA neurons was found in the different mouse mutants analyzed, recent studies support this assumption: 1) overexpression of a mutated form of *Patched1*, which successfully blocks Hh signaling (Briscoe et al., 2001; Kiecker and Lumsden, 2004), led to a suppression of DA neuron formation in the midbrain of chicken embryos (Bayly et al., 2007); 2) the DA neuronal population in the midbrain is severely reduced in the chick *talpid*^{2} mutant, which has a broad constitutive activation of the Hh signaling cascade accompanied with a reduced floor

plate (Agarwala et al., 2005); 3) ectopic expression of Shh led to an ectopic induction of DA neurons in the midbrain of chicken embryos (Watanabe and Nakamura, 2000). Furthermore, it has been shown for the mouse embryo that Shh signaling is sufficient and necessary for the generation of mesDA neurons (Hynes et al., 1997; Blaess et al., 2006). In the mouse *Shh*-null mutants a complete loss of ventral (DA and serotonergic) neuronal populations was observed in the posterior midbrain and anterior hindbrain, which may be due to the absence of the floor plate (Chiang, et al., 1996; Blaess et al., 2006), whereas in the conditional mouse *Smo-En1* knock-out, in which the Shh receptor *Smoothened* is inactivated from E8.5 onwards, these cell types are greatly reduced but still present (Blaess et al., 2006). Moreover, the induction and specification of the diencephalic DA neurons was normal in the zebrafish *smo* mutant, in which *hedgehog* signaling is also impaired, although the number of DA neurons was reduced to about half of the wild type (Chen et al., 2001; Holzschuh et al, 2003). However, these results also suggest that Hh signaling does not appear to be required for the specification of DA neurons, but rather has a function in proliferation and/or the establishment of a progenitor domain.

One very important finding of all my electroporation studies and the study by Andersson et al. (2006b) and Prakash et al. (2006) was that the ectopic induction of mesDA marker genes or mesDA neurons, respectively, only occured in the ventral aspect of the neural tube (floor and basal plate). It is therefore most likely that in addition to these factors (Wnt9a/1, Lmx1a, Shh), another ventral factor is required and/or a dorsal factor has to be repressed for mesDA neuron generation in chicken and mice.

4.4 Shh may be a regulator of *Pitx3* expression in chicken embryos

Because of the striking expression pattern of *Pitx3* in the ZLI and the complete overlap with the *Shh* expression domain in the ZLI and ventral midbrain of the chicken embryo, it was hypothesized that Pitx3 is a direct target of Shh signaling. Shh signaling is mediated by the Gli family members, which are zink- finger proteins and transcriptional targets of Shh (reviewed by Ingham and McMahon, 2001). The analysis of the Pitx3 promoter for Gli binding sites revealed that there are four binding sites for the transcription factor Zic2, which belongs to the Gli zinc-finger family (Mizugishi et al., 2001). Zic proteins can

induce or repress the expression of transcription factors, such as *En2* or *Math1*, respectively (Kuo et al., 1998; Ebert et al., 2003). Therefore, a Zic-mediated Hh signal could be responsible for the induction of the chicken *Pitx3* gene. Interestingly, electroporation of Wnt9a led to an ectopic induction of *Shh* in the ventro-lateral midbrain at the time point when ectopic *Pitx3* induction was first detected. It was therefore attempted to block Shh signaling by cyclopamine (Incardona et al., 1998) in the presence of Wnt9a, but these experiments unfortunately failed. It thus remains to be shown that Wnt9a may induce *Pitx3* via Lmx1a/Shh. The failure of the drug treatment could be due to the long incubation time of 3 days, which may have led to a dilution of the cyclopamine and a reactivation of Shh signaling, or to an inaccessibility of the target tissue. Therefore, these experiments have to be repeated.

In support of my hypothesis, a dependence of zebrafish *pitx3* expression in the ventral diencephalon on *hedgehog* signaling has been demonstrated recently (Zilinski et al., 2005). Analysis of the zebrafish *slow-muscle-omitted* mutants (*smo*) (Barresi et al., 2000; Chen et al., 2001; Varga et al., 2001), in which *hedgehog* signaling is distorted due to a mutation in the smoothened receptor, revealed a complete lack of *pitx3* expression in the anterior pituitary and ventral diencephalon, whereas expression in the lens placode was unchanged (Zilinski et al., 2005). These findings suggest that Pitx3 could indeed be regulated by Shh signaling in zebrafish and chicken. However, the comparison of the *Pitx3* promoter and 5´UTR region between chicken and other vertebrates showed that this region is only hardly conserved (3-5%) across species and that the Zic binding sites are not conserved in mice and humans, suggesting that *Pitx3* is not regulated by Hh signaling in these mammalian species, in line with previous findings (Prakash et al., 2006).

4.5 Direction of future experiments concerning mesDA neuron development in the chicken embryo

The comparison between two or more animal models will help to establish conserved pathways in the generation of mesDA neurons in vertebrates. Therefore, gain- and loss-of-function studies for these factors have to be done to address the question of the functional roles of Pitx3, Nr4a2 and Lmx1b in the development of mesDA neurons in the chicken embryo. The finding that *Pitx3* is very likely a target gene of Shh signaling in the chicken

embryo should be confirmed conclusively by repeating the cyclopamine experiments and doing *Pitx3* promoter assays (e.g. ChIP, reporter assays).

In addition, a precise temporal dissection (1d and 2d incubations) of the different electroporation experiments (Wnt9a, Lmx1a and Shh) has to be done to unravel the genetic network. As a long-term goal new downstream targets of Wnt9a/Lmx1a have to be identified which are responsible for the early induction of the mesDA neurons in the chicken embryo. Candidate target genes can be identified by bioinformatics analysis with the prediction of binding sites in the promoter region and microarray analysis after the overexpression of Wnt9a or Lmx1a, respectively.

Furthermore, long-term incubation experiments have to be done to demonstrate that ectopically induced *Pitx3+/Nr4a2+* cells after Wnt9a electroporation will also express later *Th*. For these experiments it is feasible to use the transposon-mediated gene transfer technique that enables an electropoated gene to be stably integrated into the chicken genome (Sato et al., 2007). With this technique it is possible to express the transgene at least up to 10 days, thus bypassing the dilution/functional inactivation of the plasmids.

5 Materials and methods

5.1 Laboratory equipment

Autoclave:	ASS and Aigner, Typ 667-1ST
Benchtop shakers:	Heidolph, Unimax 2010, Polymax 1040;
Benchtop thermostats:	Eppendorf, Thermomixer 5463/Comfort
Cameras:	Zeiss, AxioCam MRC, HRC; Fuji, HC2000
Centrifuges:	Sorvall, RC5C Plus (Rotors: GSA, SA600) Heraeus, Biofuge pico/fresco, Varifuge 3.0R Hettich, Universal 30F, Eba 12
Cryostat:	Microm, HM 560
Developing machine for X-ray films:	AGFA, Curix 60
Electrophoresis power supplies:	E-C Apparatus Inc., EC 250-90, EC3000-90 Consort, E443
Electroporator	BTX
Freezers (-20°C):	Liebherr
Freezers (-80°C):	Heraeus
Geiger counters:	Berthold, LB122
Gel documentation:	Herolab
Gel electrophoresis chambers:	MWG Biotech; PeqLab
Hybridization ovens:	ThermoHybaid; UVP, Hybridizer HB100
Hybridization tubes:	Thermo Hybaid
Incubators (bacteria):	New Brunswick, Innova 4230
Incubators (eucaryotic cells)	Heraeus
Isotope counter:	Scott - lab, Easicount 400
Laminar flows:	Nunc, Laminar Flow Workstation, Microflow Safety Cabinet
Light sources:	Leica, KL 1500; Zeiss, KL 2500 LCD
Magnetic stirrers:	IKA Labortechnik, RCT basic
Microscopes:	Leica DMIL, MZ8, MZ6, APO, M3Z Zeiss Axiovert 200M, Axioplan 2, Stemi SV 6, LSM 5 META
Microtomes:	Microm, HM 355S; Leica
Microwave oven:	Sharp

Paraffin embedding station:	Sakura, Tissue-TEK® TEC
PCR machines:	Eppendorf, Mastercycler Gradient
pH indicator:	WTW, pH 538
Photometer:	Eppendorf, Biophotometer
Pipettes (10µl - 1ml):	Gilson
Pipetting aids:	Brand, Accujet®
Precision balances:	Sartorius LC220S, LC6201S, BA210S
Refrigerators:	Liebherr
Stator disperser:	IKA Labortechnik, Ultraturrax
Thermomixers:	Eppendorf
Ultra pure water purification system:	Millipore, MilliQ
	Brand, Nutator
UV-DNA/RNA-Crosslinker :	Stratagene, UV Stratalinker ®1800
Vortexers:	Scientific Industries, Vortex Genie
Waterbaths:	Julabo U3 ; Lauda A100/E100; Leica HI 1210

5.2 Suppliers of enzymes, chemicals, kits and other consumables

The suppliers of materials and kit systems are cited with the corresponding method. Laboratory glass ware was obtained from Schott. Unless otherwise stated, all chemicals were supplied by Sigma-Aldrich. Restriction enzymes, T3, T7, SP6 RNA polymerases, RNase inhibitors and yeast tRNA were obtained from Roche Diagnostics.

Miscellaneous:

Ampuwa® water	Fresenius AG, Bad Homburg
Autoradiography films (Biomax, X-OMAT), X-ray cassettes	Amersham, Freiburg
Bacteria culture dishes, disposable pipettes	Greiner Labortechnik, Frickenhausen
Consumables for cell culture	Nunc, Wiesbaden
Developer (D-19), Fixer	Kodak, New Haven, USA
DNA standard for gel electrophoresis (Smartladder®)	Eurogentec, Köln
Embedding molds	Polysciences, Peel-A-Way
Falcon® tubes, canulas, syringes	Becton Dickinson, Europe (Schubert & Weiss, München)
Nuclear fotoemulsion (type NTB-2)	Kodak, New Haven, USA

Parafilm®	American National Can, USA
Pipette tips 0.1-10µl; 1-20µl; 1-200µl; 101-1000µl	Starlab, Ahrensburg
Safe-Lock reaction tubes (1.5/2.0ml)	Eppendorf, Hamburg
X-ray films:	RM2155, Kodak

5.3 Working with deoxyribonucleic acids (DNA)

5.3.1 Cleavage of plasmid DNA by restriction endonucleases

Plasmid DNA was cleaved by commercially available restriction endonucleases (RE). The amount of enzyme per µg DNA was 1-10 units. Conditions were set up according to manufacturer's recommendations and the reaction was incubated for at least 1h.

5.3.2 Dephosphorylation of linearized plasmids

To prevent the religation of a linearized plasmid, the 5' ends are dephosphorylated. Therefore, the plasmid was incubated with shrimp alkline phosphatase (USB Corporation) at 37°C for 30min. The enzyme was added directly after the restriction digest. After the reaction, the plasmid was isolated either by isopropanol precipitation (see 5.3.4.1) or by gel extraction (see 5.3.4.2).

5.3.3 DNA gel electrophoresis

Depending on the fragment sizes to be separated, TAE/agarose gels containing 0.8% (separation of large fragments up to 10kb) to 2% agarose (separation of small fragments 100-200bp) were used. DNA fragments were visualised by adding 1µg/ml ethidium bromide to the gel, which intercalates into the DNA and fluoresces at 312nm and 366nm of UV light.
6x sample buffer was added to the DNA samples, the samples were loaded on the gel and the fragments were separated in a 60-100V electric field.

50x TAE: 1.5M Tris base
1M acetic acid
0.05M EDTA
pH 8.5

6x sample buffer: 0.2% bromophenol blue
60% glycerol

5.3.4 DNA isolation

5.3.4.1 Isopropanol precipitation of DNA

DNA was precipitated from aqueous solutions by adding 1/10 volume of 3M sodium acetate (pH 5) and 1 volume isopropanol. After inverting the tube several times, the DNA precipitated as a filamentous pellet. The sample was centrifuged for 1min at 16000g, washed with 70% ethanol and centrifuged again for 1min at 16000g. The ethanol was removed with a pipette, DNA was air dried for 5-10min and redissolved in TE buffer or aqua dest.

TE buffer: 10mM Tris-HCl pH 7.5-8.0
1mM EDTA

5.3.4.2 Gel extraction of DNA fragments

The isolation of DNA fragments after gel electrophoresis was carried out using the QIAquick® Gel Extraction Kit (Qiagen) according to manufacturer's specifications. The DNA fragment of interest was excised from the gel and purified following the protocol of the kit.

5.3.4.3 Purification of PCR products

PCR products were isolated using the QIAquick® PCR Purification Kit (Qiagen) according to manufacturer's instructions.

5.3.5 Determination of DNA and RNA concentration

DNA and RNA concentration was measured using the BioPhotometer (Eppendorf) following manufacturer's instructions. To estimate the quality of the DNA, the ratio of absorbance at 260nm and 280nm was determined. A_{260}/A_{280} values ≥ 1.8 were obtained for purified DNA.

5.3.6 Ligation of DNA fragments

To covalently bind two DNA ends, T4 DNA ligase was used to create a new phosphodiester bond between the two ends, of which at least one has to carry a 5' phosphate group. Between 20-100ng of vector and a 3 – 5 fold molar excess of insert were applied in a ligation reaction. The Rapid DNA Ligation Kit (Roche Diagnostics) was routinely used to carry out DNA ligations.

Ligation reaction:
2µl 5x dilution buffer
xµl vector
yµl fragment
ad 10µl aqua dest.

The reaction set up was heated 5min to 70°C and cooled down on ice.

10µl 2x reaction buffer
1µl T4 DNA Ligase

were added and incubated 5-15min at RT. The resulting ligation product was used to transform chemically competent bacteria (5.5.3)

5.3.7 TOPO-TA Cloning®

TOPO TA Cloning® (Invitrogen) is designed to clone PCR fragments into a TOPO-Isomerase vector. The TOPO TA Cloning® kit (Invitrogen) was used following the manufacturer's protocol. The pCRII TOPO vector provided in the kit was used for cloning and the ligation product was transformed into One Shot® TOP 10 competent cells as described below (see 5.5.3).

5.3.8 DNA amplification by polymerase chain reaction (PCR)

Specifications of a routinely used PCR set up:

PCR reaction:	2µl	10x RedTaq® buffer (Sigma-Aldrich; contains $MgCl_2$)
	1µl	template (~50-200ng genomic DNA, 10ng plasmid DNA)
	0.5µl	dNTP (10 mM each)
	1µl	forward primer (10µM)
	1µl	reverse primer (10µM)
	0.25µl	RedTaq® DNA polymerase (5U/µl, Sigma-Aldrich)
	ad 20µl	aqua dest.

A list of primers is given in appendix 6.1. The PCR reactions were run in Eppendorf Mastercycler Gradient PCR machines. First, the samples were heated to 94°C for 2-4min to separate the DNA strands. Then a defined number of cycles were run dependent on the specific fragments that were amplified. In a routinely used PCR program cycles consisted of strand separation at 94°C for 30s, annealing of the primers for 30-60s (annealing temperature depends on the primers, ~5°C below melting temperature of the primers) and elongation of the primed DNA strands at 72°C for 30s to several minutes (dependent on the length of the fragments). A final elongation step at 72°C for 5-10min followed the cycles. At the end of the program samples were kept at 4°C.

5.3.9 DNA sequencing

DNA sequencing was carried out by the following companies:

Sequiserve
Bahnhofstr. 30
85560 Vaterstetten
Germany

SEQLAB
Hannah-Vogt-Str.1
Göttingen
Germany

5.4 Working with ribonucleic acids (RNA)

Handling of RNA was carried out wearing gloves and using baked glass ware or RNase free disposable plastic materials at all times. Aqua dest. was treated with diethylopyrocarbonate (DEPC) and autoclaved twice prior to use.

5.4.1 Isolation of total RNA

5.4.1.1 Isolation of total RNA from chicken tissue

Chicken tissue (embryos) was homogenized in 1ml TRIZOL® reagent (Invitrogen) per 100mg of tissue using a power homogenizer (Ultraturrax) and incubated at RT for 15min. 0.2ml chloroform per ml TRIZOL® reagent was added and the sample was shaken vigorously for 15s. After an incubation of 2-3min at RT, the sample was centrifuged at 12000g for 15min at 2-8°C. Following centrifugation, the mixture separated into a lower red phenol-chloroform phase and an upper colourless aqueous phase containing the RNA. The aqueous phase was transferred to a new reaction tube and the RNA was precipitated by adding 0.5ml isopropanol per ml TRIZOL® reagent used for the initial homogenization. Samples were incubated at RT for 10min and afterwards centrifuged at 12000g for 10min at 2-8°C. The supernatant was discarded, the RNA was washed with 70% ethanol, air dried and redissolved in the appropriate amount of DEPC-treated water.

5.4.1.2 Synthesis of mRNA

For the synthesis of capped mRNA 15µg of an expression vector coding for a gene of interest, carrying a phagepromoter at the 5`-end and a polyA sequence at the 3`-end, are linearized by digestion with an appropriate restriction enzyme. This template is then purified using the QiaQuick reactionsystem "Nucleotide Removal Kit". To ensure that the restriction digest and the purification procedure worked properly an aliquot is analysed by gel electrophoresis.

For the in-vitro transcription the Ambion „mMessage mMachine SP6 Kit" was used. The

reaction was performed according to the manufacturers specifications for 2-3h at 37°C with the following volumes:

linearized template-DNA	6µl
2x NTP/CAP	10µl
10x SP6-polymerase reaction buffer	2µl
enzyme-mix (SP6)	2µl

Afterwards 1.5µl RNase-free DNaseI (1U/µl) is added to the reaction and the sample is incubated for 20 min at 37°C. The synthesized mRNA is purified using the Qiagen „RNeasy Mini Kit“ according to the specifications of Qiagen. The RNA is eluted in 50 µl water.
For further purification and concentration 1/10 of the reaction volume of 3M NaOAc and 3 times the reaction volume of EtOH is added and the sample is incubated for 20min at –20°C. To precipitate the mRNA the sample is centrifuged at maximum speed (Centrifuge 5415D, Eppendorf, 13200rpm) for 30min. The supernatant is discarded and the RNA pellet washed in 70% EtOH and centrifuged at maximum speed for 15min at 4°C (Centrifuge 5415D, Eppendorf, 13200rpm). The supernatant is discarded again and the pellet is dried and resuspended in 20µl water.
The yield as well as the integrity of the synthesized mRNA is controlled by gel electrophoresis and the concentration of the obtained mRNA solution is determined by measuring the OD_{260}.

The mRNA can be stored at –20°C for several months.

5.4.1.3 Gel electrophoresis of RNA

For the electrophoretic separation of RNA fragments, 1.5% agarose gels containing formaldehyde and a special running buffer were used. 20µl loading buffer were added to 5µl RNA samples (~1.5µg/µl), samples were heated to 95°C for 5min, vortexed briefly and loaded onto a 1.5% agarose gel. Gels were run at 70 – 80V for 1-1,5h.

10x running buffer: 200mM MOPS
50mM sodium acetate
10mM EDTA
pH 7.0

1.5% gel: 1.5% agarose
10% 10x running buffer
0.025mM ethidium bromide (10mg/ml)
0.6% formaldehyde solution
in DEPC-treated water

Loading buffer: 0.025% bromophenol blue
40% formamide
7.4% formaldehyde
20% 10x running buffer

5.4.2 cDNA synthesis by reverse transcription

Single stranded cDNA was synthesized from total RNA (see 4.4.1) using the Advantage™ RT-for-PCR Kit (BD Biosciences, Clontech) following the manufacturer's instructions. A standard reaction yielded a 100µl cDNA sample, of which 2-5µl were used as template in a PCR setup.

5.5 Working with *Escherichia coli*

5.5.1 Storage of bacteria

For the long term storage of bacteria, glycerol stocks were prepared containing 250µl 80% glycerol in PBS and 750µl bacterial suspension from a 100ml over night culture. The glycerol stocks were stored at -80°C.

5.5.2 Preparation of chemically competent cells

Bacteria from a glycerol stock were streaked out onto an LB agar plate and incubated over night at 37°C. 5ml of LB medium were inoculated with a single colony and incubated over night at 37°C on a shaker. 1ml of this culture was used to inoculate 100ml of LB medium,

which was incubated at 37°C on a shaker until the suspension had reached an OD_{600} of 0.3-0.4. The culture was centrifuged at 2000g for 10min at 4°C and resuspended on ice in 10ml TSS. 100-200µl of competent cells were used for a standard transformation (see 5.5.3).

1x LB medium: 0.1% Bactotrypton (Difco)
0.5% Bacto-Yeast-Extract (Difco)
0.17M NaCl
pH 7.0

TSS: 50% 2x LB medium (autoclaved)
10% polyethylenglycol (PEG)
5% DMSO
0.05M $MgCl_2$ (autoclaved)

LB-Agar: 1x LB medium
1.5% agar (Bacto-Agar, Difco)

5.5.3 Chemical transformation of bacteria

100-200µl of chemo-competent cells were thawed on ice and 1ng (retransformation) - 50ng (ligation reaction) DNA were added. The cells were incubated on ice for 30min, then heat shocked for 30s at 42°C in a water bath and transferred back on ice for 2min. 500µl of RT LB medium were added and the cells were incubated for 30-60min at 37°C on a shaker. 50-200µl of the bacterial suspension were plated on LB agar plates containing an appropriate antibiotic for selection (Ampicillin (100µg/ml), Kanamycin (50µg/ml), Chloramphenicol (25µg/ml)) and the plates were incubated at 37°C over night. Single colonies were used to inoculate 2-5ml LB medium cultures, which were incubated over night at 37°C on a shaker. After isolation of the plasmid DNA (see 5.5.4), transformant clones were identified by PCR (see 5.3.8) and/or restriction digest (see 5.3.1).

5.5.4 Isolation of plasmid DNA from *E. coli*

Depending on the amount of bacterial culture, so called Mini- (3-5ml), Midi- (50-100ml) or Maxi-Preparations (200-1000ml) for the isolation of plasmid DNA were carried out using the following kits:

QIAprep® Miniprep Kit (Qiagen)
QIAGEN® Plasmid Midi Kit (Qiagen)
QIAGEN® Plasmid Maxi Kit (Qiagen)
QIAGEN® HiSpeed Maxi Kit (Qiagen)
Wizard® *Plus* Midipreps DNA Purification System (Promega)

5.6 Animal handling

All fertilized hens´eggs were supplied from a local farm (Brüterei Ludwig Hölzl). Eggs were stored at room temperature and incubated in a humidified atmosphere at 38°C until they had reached the desired stage.
Zebrafish (Danio rerio) were raised and maintained on a 14/10 hours light/dark cycle. The embryos were obtained by natural spawning and raised at 28.5°C. Wildtyp-zebrafish strains used were the AB-strain (inbred strain at the GSF National Research Centre for Environment and Health) and Brass (inbred strain of pigmentation mutant that is deficient in melanine synthesis at the GSF National Research Centre for Environment and Health).

5.6.1 Determination of embryonic stages

In general, chicken embryos were staged according to Hamburger and Hamilton (HH; 1992) or embryonic days (E) with E1 meaning one day of incubation.
The developmental stage of zebrafish embryos was determined by morphological criteria after Kimmel et al. (1995) and indicated as either hours or days post-fertilization (hpf or dpf).

5.6.2 *In ovo* electroporation

Chick embryos were incubated until the desired stage (HH10-12 or HH16/17). The eggs were desinfected with 70% ethanol and 4 ml of albumen were removed with a syringe. Chicken eggs were "windowed" for manipulation and prepared for electroporation by making an incision with a sharpened tungsten needle in the vitelline membrane at the level of the anterior neural tube. Plasmid DNA (2-3µg/µl) was mixed 1:20 with 0,1% fast green

(Sigma) for visualization of the injected DNA. The electrodes were fixed on a micromanipulator and placed lateral and parallel along the anterior-posterior axis of the midbrain with a distance of 5mm (Nakamura and Funahashi, 2001). The tip of the microcapillary was inserted into the lumen of the midbrain, and the DNA was injected using a microinjector. Five times pulses with 7-15V, each of 50 msec duration, were generated by an BTX electroporator. The DNA was driven into the neural tube cells that lie adjacent to the anode, whereas cells on the cathode side served as control. After electroporation a drop of sterile cold PBS with 1% Penicillin/Streptomycin (Invitrogen) was placed onto the embryo. Then the window in the eggshell was sealed with tissue tape and the injected embryos were reincubated at 37°C for 1-5 days.

5.6.3 **RNA-cytoplasmic injection** (Ekker et al., 1995)

For cytoplasmic injections one cell stage zebrafish eggs are lined up in petri dishes filled with agarose (1.5% agarose/ 30% Danieau`s solution) in which a mold was embedded to obtain grooves.

In vitro synthesized mRNA is diluted in water to the desired injection concentration with water (typically 100-250 ng/ml). To facilitate the injection phenol red can be added, usually 10% of an injection mix volume of 10-15 ml.

A glass capillary needle is filled with the injection mix and positioned into a micromanipulator. With the help of a binocular the chorion and the cellular membrane of the embryo are penetrated with the needle and mRNA (500pg to 2ng) is injected into the cytoplasm. After the injection the embryos are seperated spatially to allow an even oxygen supply which is necessary for synchronous development. The embryos are incubated at 28°C until they reach the desired development stage and then fixed in 4% paraformaldehyde (PFA).

300% Danieau-solution :

58 mM NaCl
0,7 mM KCl
0,4 mM $MgSO_4$ x $7H_2O$
0,6 mM $Ca(NO_3)_2$
5 mM HEPES (pH 7.2)

5.6.4 Dissection of chicken embryos

Fertile hens´ eggs were incubated horizontally in a humidified atmosphere at 38°C to the required stage, desinfected with 70% ethanol and carefully opened into a petridish. The embryos were dissected out from the yolk with a pair of scissors and transferred into PBS. The embryos were freed from vitelline membrane and other embryonic membranes and fixed in 4% PFA in PBS over night at 4°C. The next day embryos were processed either for paraffin or cryo embedding or for whole mount *in situ* hybridisation.

1x PBS:
137mM NaCl
2.7mM KCl
10mM Na_2HPO_4
2mM KH_2PO_4
pH 7.4

5.6.4.1 Paraffin embedding of embryos

For paraffin sections, embryos were dehydrated in a graded ethanol series (70%, 96%, 100%) and cleared in xylene. Times for each dehydration step varied with embryonic stage (see table 1). For storage, embryos were kept in 70% ethanol at RT. Incubation times in xylene may vary and had to be checked for transparence of the embryos.

Embryonic stage	70% ethanol	96% ethanol	100% ethanol	xylene
E2.25	> 2h	10min	10min	5min
E3	> 2h	10min	10min	10min
E3.75	> 2h	15min	15min	10min
E5.5	3-4h	15min	15min	10-15min
E7.25	3-4h	20min	20min	15min
E8.5	> 4h	30min	30min	20-30min

Table 1 Incubation times for dehydration and clearance of mouse embryos at different stages and for adult brains.

After clearing, the embryos were transferred into liquid paraffin (65°C), incubated over night at 65°C and then embedded in fresh paraffin in embedding molds.

5.6.4.2 Cryoprotection of embryos

For cryostat sections, embryos were washed in PBS for several hours after fixation over night in 4% PFA and transferred into 20% sucrose in PBS. After incubation for at least over night at 4°C, the sucrose solution was removed by using clean paper towels and embryos were embedded in TissueTek® OCT medium (Sakura) at –20°C.

5.6.4.3 Gelatine-albumin embedding

For vibratome sections embryos were incubated for several hours in stock solution. Then, embryos were transferred into plastic molds and remaining solution was removed. 5 ml of the stock solution was rapidely mixed with a mixture of 500 µl formaldehyde and 100 µl glutaraldehyde and poured over the embryos. The embryos were orientated quickly, because the solution was hardened within 10 sec.

Gelatine-albumin stock solution: 45g Gelatine dissolved in 100 ml PBS (at high T)
270g albumin
180g sucrose
ad 1L PBS
store at –20°C

5.6.4.4 Collagen gel cultures

Defined regions of the neural tube for collagen gel culture were dissected from chick embryos at stage HH14 or HH16/17 by sharpened tungsten needles (Lumsden and Davies, 1983). Rat-tail collagen was mixed with 10% BME medium (10x, Invitrogen), 20µl/ml of 7.5% bicarbonate (Invitrogen), and thoroughly mixed. A drop of collagen mix (50 µl) was placed into a 4-well plate and explants were added using a pipette and arranged as open book preparations using a sharpened tungsten needle. Gels were left to set for 15 min in the

incubator before the addition of 500 µl of F-12 medium (Invitrogen, Karlsruhe, Germany) containing 2% Sato-mix (Bottenstein and Sato, 1979). The collagen cultures were incubated for 4-5 days at 37 °C and 4% CO_2 and 50% relative humidity, then fixed in 4% PFA and used for *in situ* hybridisation.

5.7 Histological techniques

5.7.1 Sectioning of embryos

5.7.1.1 Paraffin sections

Paraffin embedded embryos and adult brains were cut on a microtome in sections of 8µm thickness. The sections were transferred to a water bath (37°C), drawn on microscope slides (Superfrost®Plus, Menzel GmbH) and placed on a heating plate (Leica) at 37-40°C. Slides were incubated over night at 37°C and stored at 4°C.

5.7.1.2 Cryosections

Embryos embedded in TissueTek® OCT medium (Sakura) were adapted to -20°C for at least 30min in the cryostat. 16µm thick sections were cut in a cryostat and transferred to cooled microscope slides (Superfrost®Plus, Menzel GmbH), placed on a heating plate (37°C) for several minutes and precooled again for the next section. Slides were stored at -20°C.

5.7.1.3 Vibratome sections

Embyos embedded in Gelatine-albumin were cut at a vibratome in sections of 30-60 µm thickness. The sections were transferred on microscope slides (Superfrost®Plus, Menzel GmbH) airdryed. Sections were then mounted with Aqua PolyMount (Polysciences, Inc.).

5.7.2 Immunohistochemistry on paraffin and cryosections

5.7.2.1 Standard immunohistochemistry on paraffin sections

All incubation steps were carried out at RT unless otherwise stated. Paraffin was removed from the sections by incubation for at least 1h in Roti®-Histol (Roth) followed by dehydration in a graded series of ethanol (2x 5min in 100% ethanol, 2x 5min in 96% ethanol, 2x 5min in 70% ethanol). Slides were rinsed for 10min in aqua dest. and then incubated for 3min in 0.01M sodium citrate, pH 6.0. For antigen retrieval, slides were boiled in a microwave (900W, 5min) in 0.01M sodium citrate, cooled down for 10min at RT, boiled again in the microwave (600W, 3min) and cooled down to RT. After washing 2x 5min in PBS, slides were incubated for 5min in 0.01% H_2O_2 in PBS to inactivate endogenous peroxidases followed by washing 2x 5min in PBS. To minimize unspecific binding of the antibody, slides were incubated in blocking solution for 1h in a humid chamber. The primary antibody was diluted in blocking solution, 100µl of antibody solution were pipetted on each slide and slides were covered with a coverslip and incubated over night at 4°C in a humid chamber. A list of applied primary and secondary antibodies with dilution factors is given in appendix 7.4.

For peroxidase-stained sections, slides were washed the next day 3x 10min in PBS and then incubated with a biotin conjugated secondary antibody diluted in blocking solution for 1h at RT followed by 3 washes for 15min in PBS. To enhance signal intensity, slides were treated with the Vectastain ABC kit (Vector Laboratories) according to manufacturer's instructions. After washing 2x 5min in PBS, slides were incubated for 5min in 0.1M Tris-HCl, pH 7.4. For the peroxidase reaction, 500µl DAB solution were pipetted on each slide and incubated 1-15min in a humid chamber, depending on signal intensity and signal to background ratio. Slides were washed 2x 5min in PBS, dehydrated in a graded series of ethanol and cleared in Roti®-Histol (Roth), after which they were coverslipped Roti®-Histokitt.

For immunofluorescence, sections were incubated with Cy2- or Cy3-conjugated secondary antibodies in blocking medium. After 3 washes for 5min in PBS, slides were rinsed briefly in aqua dest. and coverslipped in ProTaqstura (Biocyc GmbH & Co. KG).

Blocking solution: 10% Fetal Calf Serum (Gibco)
0.25% Triton-100
in 1x PBS

DAB solution: 0.05mM 3,3'-diaminobenzidine (DAB) solution
0.1 M Tris-HCl, pH 7.4
0.02% H_2O_2

5.7.2.2 Standard immunohistochemistry on cryosections

Cryosections were thawed at RT for at least 30min before use. Slides were washed 2x 5min in PBS and then treated as described in 5.7.2.1 for paraffin sections, omitting the deparaffination/rehydration steps.

5.7.2.3 BrdU labeling and immunodetection

Cells undergoing DNA replication during the S phase of the cell cycle can be labeled with the thymidine analog Bromo-2'-deoxy-uridine (BrdU) which is incorporated into newly synthesized DNA instead of endogenous thymidine. To label embryos with BrdU, embryos were injected at stage HH 16/17 with BrdU reagent into the neural tube. Embryos were dissected after 2 hours and processed for cryosectioning as described in 5.6.4.2 and 5.7.1.2. Immunohistochemistry was carried out as described in 5.7.2.2, only that the primary antibody (Bromo-2'-deoxy-uridine Labelling and Detection Kit II, Roche) was incubated for 30min at 37°C prior to over night incubation at 4°C.

5.7.3 Whole mount immunohistochemistry

After fixation in 4%PFA the embryos were washed 3x 20min in antibody solution (AS). The primary antibody was diluted in AS and the embryos were incubated at 4°C 2-4 days on a horizontal shaker. Embryos were again washed in AS several times and then incubated with the secondary antibody at 4°C over night. If a fluorochrome-coupled secondary antibody was used, all steps were then done in the dark. The antibodies were diluted according to appendix 7.4.

5.7.4 Cresyl violet staining

Paraffin sections were deparaffinated and rehydrated as described in 5.7.5.2, cryosections were washed for 1min in 50% ethanol and 70% ethanol, respectively, and then rinsed briefly in aqua dest. Sections were stained for 10-15s in cresyl violet solution, rinsed for 2min in aqua dest. and differentiated by incubation for 2x 2min in 70% ethanol, 15s in 96% ethanol containing 1% acetic acid, 2x 1min in 96% ethanol and 2x 2min in 100% ethanol. Slides were coverslipped as described in 5.7.2.1.

Cresylviolet solution: 7.8mM cresyl violet
12.5mM sodium acetate
25mM acetic acid
pH 3.5
filtrate before use

5.7.5 *In situ* hybridization on paraffin sections

For *in situ* hybridization, DEPC-treated water was used for all solutions and work was carried out wearing gloves at all times to avoid RNA degradation by RNase contamination.

5.7.5.1 Synthesis of radioactively labelled RNA probes

Radioactively labelled RNA probes (riboprobes) were synthesized by *in vitro* transcription. As templates, plasmids carrying a cDNA fragment corresponding to the designated mRNA of interest, flanked by 2 different RNA polymerase promotors, were used. The plasmids were linearized with the appropriate restriction endonuclease (see 5.3.1) either at the 5'-end (for the antisense probe) or the 3'-end (for the sense probe as control) of the cDNA fragment. The DNA was purified by isopropanol/ethanol precipitation. Probes were labelled with uridine 5'-[_-^{35}S]thiotriphosphate (^{35}S-UTP, Amersham) using the following reaction setup:

3µl 10x transcription buffer
3µl ATP/CTP/GTP ribonucleotide mix (10mM each)
1µl 0.5 M dithiothreitol (DTT)
1µl Protector RNase Inhibitor (Roche)
1.5µg linearized plasmid
3µl ^{35}S-UTP (0.1 mCi)
1µl RNA polymerase (T3, T7 or SP6)
ad 30µl aqua dest.

The reaction was incubated for 3h at 37°C, adding another microliter of RNA polymerase after 1h. To destroy the DNA template after *in vitro* transcription, 2µl of DNase I (10U/µl, Roche) were added to the reaction and incubated for 15min. Riboprobes were purified using the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's protocol. 1µl of the ^{35}S-labelled riboprobe was added to 2ml scintillation liquid (Rotiszint®, Roth) and the activity of the probe was determined in a ß-radiation counter (Easicount 400, Scott-Lab). Values between 0.5-2.5 x 10^6cpm were obtained. Riboprobes were stored at -20°C for up to 1 week.

5.7.5.2 Pretreatment of paraffin sections

All steps were carried out at RT, unless otherwise stated. Paraffin sections were deparaffinated by incubation for 2x 30min in Roti®-Histol (Roth), followed by 2x 5min in 100% ethanol and 5min in 70% ethanol. Slides were rinsed for 3min in aqua dest. and 3min in PBS and then incubated for 20min in 4% PFA in PBS on ice. The slides were washed 2x 5min in PBS, incubated for 7min in Proteinase K (20µg/ml) in Proteinase K buffer and washed again 5min in PBS. Sections were fixed again for 20min in 4% PFA in PBS on ice, washed 5min in PBS and then treated with 0.1M triethanolamine-HCl (pH 8.0) for 10min, adding dropwise 630µl acetic acid anhydrate while stirring rapidly. Subsequently, slides were washed 2x 5min in 2x SSC, dehydrated in a graded ethanol series (1min each in 60%, 70%, 96% and 100% ethanol) and air dried for 30-60min.

Proteinase K buffer: 0.5M Tris-HCl pH7.5
0.05M EDTA pH 8.0
pH to 7.8

20x SSC: 3M NaCl
300mM sodium citrate
pH to 7.4

5.7.5.3 Hybridization of pretreated slides with a riboprobe

Slides were prehybridized with hybridization mix for at least 1h at 57°C. To this end, 100µl of hybridization mix were pipetted on each slide, slides were covered with a coverslip and put into a humid chamber containing hybridization chamber fluid. For hybridization, 100µl hybridization mix per slide containing the radioactively labelled riboprobe (7 x 10^6cpm) were heated for 2min to 94°C and put on ice. Prehybridization mix was removed from the slides together with the coverslip. The hybridization mix with the riboprobe was applied and slides were covered with a new coverslip. Hybridization was carried out over night at 57°C.

Hybridization mix: 50% formamide
20mM Tris-HCl pH 8.0
300mM NaCl
5mM EDTA pH 8.0
10% dextrane sulfate
0.02% Ficoll 400
0.02% BSA
0.5mg/ml yeast tRNA
0.2mg/ml carrier DNA (salmon sperm DNA, Sigma)
200mM DTT

Chamber fluid: 50% formamide
10% 20x SSC
in aqua dest.

5.7.5.4 Stringent washes

Coverslips were removed and slides were washed 4x 5min in 4x SSC at RT. Slides were incubated for 30min in RNase A (20µg/ml in NTE buffer) at 37°C to remove excess unbound riboprobe. Slides were rinsed in decreasing concentrations of SSC buffer containing 1mM DTT (5min 2x SSC, 10min 1x SSC, 10min 0.5x SSC) at RT and then washed under high stringency conditions 2x 30min in 0.1x SSC, 1mM DTT at 64°C. After 2 washing steps for 10min in 0.1x SSC, slides were dehydrated in a graded ethanol series (1min each in 30% ethanol/300mM NH_4OAc, 50% ethanol/300mM NH_4OAc, 70% ethanol/300mM NH_4OAc, 96% ethanol, 2x 100% ethanol) and air dried for 30-60min.

NTE buffer: 0.5M NaCl
10mM Tris-HCl pH 8.0
5mM EDTA pH 8.0

5.7.5.5 Exposure of slides to autoradiographic films and nuclear fotoemulsion

The following procedures were carried out in a dark room. To check whether the hybridization procedure was successful and to determine the intensity of the signal, slides were exposed to an X-ray film for 2-3 days. Slides were then dipped in KODAK NTB2 emulsion (Kodak). The fotoemulsion was prewarmed in a waterbath to 42°C and diluted 1:1 with prewarmed aqua dest. Slides were inserted into plastic mounts and slowly immersed into the emulsion. Excess emulsion was blotted on a paper towel and the slides were left drying over night. Slides were then sorted into dark plastic boxes containing silica gel desiccant and exposed for 4-6 weeks at 4°C.

5.7.5.6 Development of slides

Slides were equilibrated to RT for at least 2h before proceeding with development. Slides were developed for 4min in KODAK D19 developer (Kodak), washed for 30s in tap water and fixed for 15-20min in KODAK fixer (Kodak). Slides were rinsed for 15-20min in

flowing tap water. After air drying, remaining fotoemulsion was removed from the back of the slides using a razor blade. Slides were routinely stained with cresyl violet (see 5.7.4).

5.7.6 Whole mount *in situ* hybridization

5.7.6.1 Synthesis of digoxigenin (Dig)- and fluorescein (Fluo)-labelled RNA probes

RNA probes for whole mount *in situ* hybridization (WISH) were synthesized similar to radioactively labelled probes, using the same linearized plasmids as templates for *in vitro* transcription. Instead of uridine 5'-[a-35S]thiotriphosphate, digoxigenin-11-uridine-5'-triphosphate (Dig-UTP, Roche) or fluorescein-12-uridine-5'-triphosphate (Fluo-UTP, Roche) were incorporated into the RNA molecule. The reaction was set up with the following components:

	8 µl 5x transcription buffer
	4 µl ribonucleotide mix (see below)
	2 µl Protector RNase Inhibitor (Roche)
	1.5 µg linearized plasmid
	2 µl RNA polymerase (T3, T7 or SP6)
	ad 40 µl aqua dest.
ribonucleotide mix:	2 µl 100 mM ATP
	2 µl 100 mM GTP
	2 µl 100 mM CTP
	1.3 µl 100 mM UTP
	7 µl 10 mM Dig-UTP/Fluo-UTP
	ad 20 µl aqua dest.

The reaction was incubated for 2 hours at 37°C, then 1 µl DNase I (10 units/µl, Roche) was added. Enzymes were inactivated by adding 1.6 µl 0.5 M EDTA pH 8.0 and the probe was purified using the RNeasy MinElute Cleanup Kit (Qiagen) following the protocol. The integrity of the probe was checked by running 2 µl of the eluate on a 1.5 % TAE/agarose gel under RNase-free conditions, using RNA loading buffer (see 5.4.1.3).

5.7.6.2 Dot blot

To estimate the labelling efficiency of the transcription reaction, a dot blot was performed. Serial dilutions of RNA probes and of Dig-labelled control RNA (100 ng/µl, Roche) were prepared using RNA dilution buffer. Dilutions between 1:10 and $1:10^6$ were set up. 1 µl of each dilution was pipetted on a rectangular piece of Hybond N membrane (Amersham), marking each spot with a pencil. RNA was crosslinked to the membrane by UV light (1200 J, 60 seconds). The membrane was washed 2x 5 minutes in buffer 1, blocked for 30 minutes in blocking buffer and incubated for 30 minutes with an alkaline phosphatase-conjugated anti-Dig and/or anti-Fluo antibody (diluted 1:5000 in blocking buffer, Roche). After washing 2x 15 minutes in buffer 1, the membrane was incubated 5 minutes in buffer 3 and then stained in staining solution in the dark for approximately 30 minutes. The staining reaction was stopped by incubation for 5 minutes in buffer 4, the membrane was rinsed in aqua dest. and air dried. Dig- or Fluo-UTP incorporation was estimated by comparison of the limit for detection of control RNA and synthesized probe.

RNA dilution buffer: 500 µl aqua dest.
300 µl 20x SSC
200 µl formamide

Buffer 1: 100 mM Tris-HCl pH 7.5
150 mM NaCl

Blocking buffer: buffer 1
1% blocking reagent (Roche)

Buffer 3: 100 mM Tris-HCl pH 9.5
100 mM NaCl
50 mM $MgCl_2$

Staining solution: 10 ml buffer 3
45 µl NBT (75 mg/ml in 70% DMF, Roche)
35 µl BCIP (50 mg/ml in 100% DMF, Roche)

Buffer 4: 10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0

NBT = 4-Nitro blue tetrazolium chloride
BCIP = 5-bromo-4-chloro-3-indolyl-phosphate

5.7.6.3 Fixation of embryos for WISH

Chicken embryos were dissected and fixed in 4% PFA in PBS as described in 5.6.4. Embryos were processed in 24 well-plates (Nunc). All incubation steps were carried out at RT on an orbital shaker, unless otherwise stated.
Following incubation in 4% PFA, embryos were washed 3x 5 minutes in PBT and then dehydrated by a graded series of methanol (5 minutes in 25% methanol in PBT, 50% methanol in PBT, 75% methanol in PBT, 100% methanol, respectively). Embryos were stored at -20°C in 100% methanol (-80°C for longterm storage).

PBT:
5 mM NaH_2PO_4
5 mM Na_2HPO_4
2.5 mM KCl
137 mM NaCl
0.1 % Tween-20
pH 7.4

5.7.6.4 Pretreatment and hybridization of embryos

Embryos were bleached for 60 minutes in 6% H_2O_2 in methanol, rehydrated in a graded series of methanol (5 minutes in 75% methanol in PBT, 50% methanol in PBT, 25% methanol in PBT, respectively) and washed 5x 5 minutes in PBT. They were treated for 3x 20 minutes with Detergent Solution, followed by fixation in 0.25% glutaraldehyde, 4% PFA in PBT. After washing 3x 5 minutes in PBT, embryos were prehybridized in prewarmed hybridization buffer for at least 1 hour at 70°C without shaking. Hybridization was carried out over night in hybridization buffer containing 0,5 - 1 µg labelled probe at 70°C without shaking. Before use, the hybridization buffer with the probe was heated to 70°C for 10 minutes and cooled down to room temperature.

Detergent Solution:
1% Nonidet P-40
1% SDS
0,5% Deoxycholate
50 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0
150 mM NaCl

Hybridization buffer: 50% formamide
5x SSC
2% SDS
50 µg/µl heparan sulphate
50 µg/ml yeast tRNA
2% BBR

5.7.6.5 Washing of embryos and detection of Dig-/Fluo-labelled RNA probes

Embryos were washed 4x 30 minutes in preheated Solution X at hybridization temperature without shaking. After washing 3x 10 minutes in MABT, they were incubated in blocking solution for at least 1 hour and then incubated with alkaline phosphatase-conjugated anti-Dig antibody (diluted 1:2000 in blocking solution, Roche) over night at 4°C. The next day, embryos were rinsed briefly 3x in MABT and washed 6x 60 minutes in MABT followed by a final washing step in MABT at 4°C over night.

Solution X: 50% formamide
2x SSC pH 4.5
1% SDS

MABT: 100 mM maleic acid
150 mM NaCl
0.1% Tween-20
pH 7.5

Blocking solution: 2% BBR (Roche)
20% normal sheep serum (NSS)
in MABT

5.7.6.6 Staining of embryos

Embryos were washed 3x 10 minutes in NTMT and then stained in fresh staining solution in the dark on a shaker. Staining was checked regularly and the reaction was stopped by transferring the embryos to PBT. They were washed 2x 10 minutes in PBT and then fixed in 4% PFA. Embryos were either stored at 4°C, or subjected to a second round of

immunodetection for double labeled embryos. Detection of Fluo-labelled RNA probe was carried out as described for Dig-labelled probe, using an alkaline phosphatase-conjugated anti-Fluo antibody (1:3000, Roche) and staining with INT/BCIP (75 mg/ml) in NTMT.

NTMT: 100 mM NaCl
100 mM Tris-HCl pH 9.5
50 mM $MgCl_2$
0,1% Tween-20

INT = 2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium chloride (Roche)

5.8 Microscopy and image editing

Brightfield and darkfield microscopy was carried out on a Zeiss Axioplan2 microscope (5x-100x Plan Neofluar® objectives, Zeiss) or on a Zeiss Stemi SV 6 stereomicroscope (Plan-achromat S objective, Zeiss). For fluorescence microscopy, the Zeiss Axiovert 200M and the confocal microscope Zeiss LSM 510 META were used. Images were taken with the digital cameras Zeiss AxioCam MRC/HRC and recorded using Axiovision software, versions 3.1 and 4.0. If necessary, images were edited with Adobe Photoshop software, versions 7.0.

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7. Appendix

7.1 Primers for PCR

Primers for cloning full length cDNA clones

Pitx3 (accession number: ENSGALG9008; XM_421631)

forward	reverse
5′-CACCACCACCCATTCCTGC-3′	5′-GTCCCCAGTTAGCAGAGAG-3′

Wnt5a (accession number: NM_204887)

forward	reverse
5′-GATTGCTGATGGACCTTGGA-3′	5′-AGTTCCTCCCACCTCGTCC-3′

Wnt6 (accession number: NM_001007594)

forward
5′-GCGCGCTCTAGAATGGACCCCAACAGCATC-3′
reverse
5′-GCGCGCGAATTCTCAGATGCAGAGGCTGAG-3′

Wnt7a (accession number: NM_204292)

forward
5′-GCGCTCTAGAATGAACAGGAAAACAAGG-3′
reverse
5′-GCGCGCGAATTATCACTTACAGGTATATAC-3′

Wnt9a (accession number: ENSGALT00000008675; NM_204981)

forward	reverse
5′-CGCCGCTGCAAGATGCTG-3′	5′-GGTTCGCATCAGGCAGAACA-3′

Primers for cloning the POU-HD of Pitx3

Pitx3_for_XhoI
5′-GACCTCGAGCTCAAGAAGAAGAAGCAGCGGAGGCAGCG-3′

Pitx3_rev_SpeIXbaI
5′-CTCTCTAGAACTAGTCCTCTCCCGTTTCCTCCACTTAGCCC-3′

Pitx3_rev_SalIXbaI
5′- TCATCTAGAGTCGACCTCTCCCGTTTCCTCCACTTAGCCC-3′

Primers for sequencing

pMES_for1
5′-CATGCCTTCTTCTTTTTCC-3′

pMES_for3
5′-TTCGGCTTCTGGCGTGTGA-3′

pMES_rev1
5′-GCCAGGTTTCCGGGCC-3′

pMES_rev4
5′-GTTTCCGGGCCCTCACATT-3′

pCS2_SP6
5′-CCCAAGCTTGATTTAGGTGAC-3′

pCS2_T7
5′-AATACGACTCACTATAG-3′

Primers for cloning in situ probes

cAldh1a3 (accession number: NM_204669.1)
cRaldh3_for
5′-CTGGAGCAGCCATCTCTACC-3′

cRaldh3_rev
5′-AACACTGCAGCTGTGAGTCC-3′

cLmx1a (accession number: NM_033652)
cLmx1a_for
5′-GCTGGACGGCTTGAAGATG-3′

cLmx1a_rev
5′-GGTGAAGTAGGAGCTCTGC-3′

cPatched1 (accession number: NM_204960)
cPatched1_for
5′-CACAGAAAGCAGACTATCC-3′

cPatched1_rev
5′CTTCCCTTCAGTTACCATC-3′

7.2 Plasmids used for electroporation

7.2.1 pMES vector (Chen et al., 2004)

The pMES expression vector was used for cloning the cWnt9a and cShh. The gene of interest will be transcribed at the same time with an eGFP reporter from a single bicistronic mRNA, which is achived by an internal ribosome entry side (IRES) of the *Encephalomyocarditis Virus* (ECMV), situated between a multiple cloning side (MCS) and the eGFP coding region (Fig. 35). The expression level of eGFP is equivalent to that of the gene of interest. Efficient ubiquitous expression of the gene of interest is achieved by the use of a *Cytomegalovirus* (CMV) enhancer fused to a chicken ß-actin promoter. The rabbit ß-globin 3′UTR includes the poly(A) motif. This vector was kindly provided by Christine Krull.

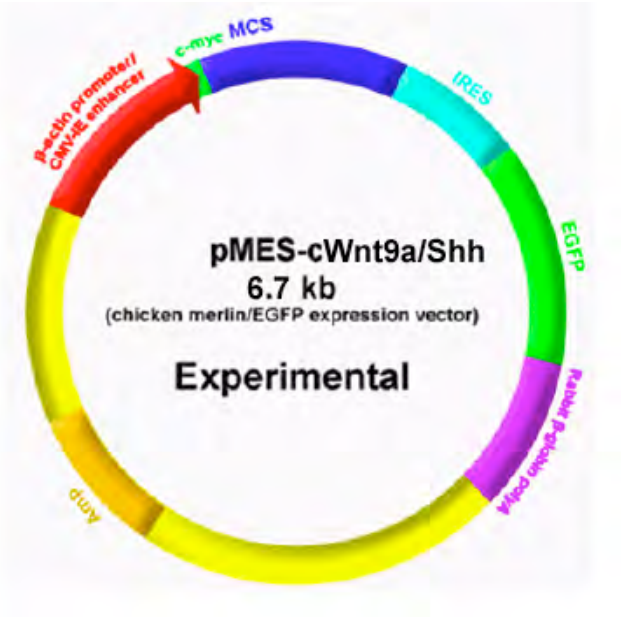

Figure 35: Map of the pMES vector used for the electroporations. For details see text.

7.2.2 **pCAX vector** (Chen et al., 2004)

The pCAX expression vector was eithet used for co-injection with pECE or as control. This vector derives from pCAGGS vector with mGFP6 inserted downstream from the CAG promoter/CMV-IE enhancer (Fig. 36). The mGFP6 gives a brighter fluorescence in eukaryotic cells. This vector was a kind gift from John Guildford.

Figure 36: Map of the pCAX expression vector. For further details see text.

7.2.3 **pECE vector** (Ellis et al., 1986)

The cDNA of mLmx1a was cloned into the pECE vector (Fig. 37), as previously described in Andersson et al. (2006b) and kindly provided by Johan Ericson.

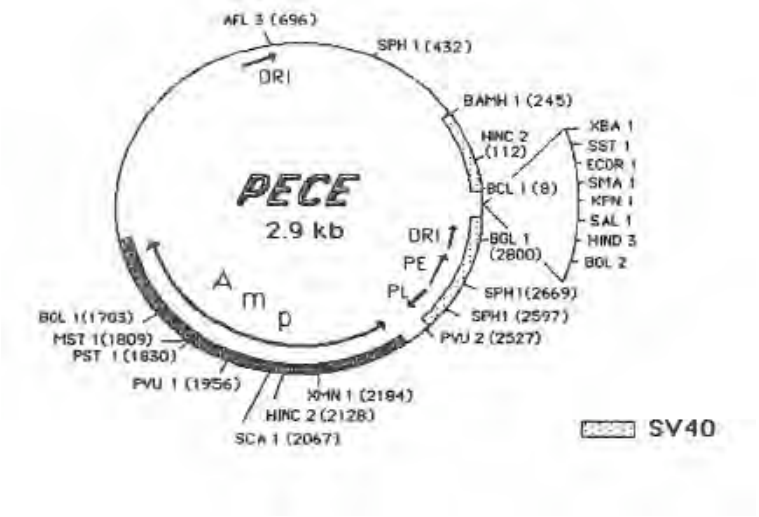

Figure 37: Map of the pECE vector. For further details see Andersson et al. (2006b).

7.3 In situ probes

Template	Linearized with	Transcribed with	Provided by	Accession number
cAldh1a1	NotI	T3	BBRSC	NM_204577.3
cAldh1a2	NotI	T3	BBRSC	NM_204995.1
cAldh1a3	BamHI	T7	R. Klafke	NM_204669.1
cEn1	NotI	T3	BBRSC	XM_001234328
cFgf8	EcoRI	T7	G. Martin	NM_001012767
cLmx1a	NotI	SP6	R. Klafke	XM_001236605.1
mLmx1a	NotI	SP6	S. Laaß	NM-033652
cLmx1b	HindIII	T3	H. Nakamura	NM_205358
cMsx1	BglII	T3	R. Wingate	NM_205488.1
cNkx2.2	SpeI	T7	J. Rubenstein	AF110994

cNkx6.1	EcoRI	T7	J. Rubenstein	AF102991
cNgn1	EcoRI	T7	H. Domingos	NM_204883.1
mNr4a2	HindIII	T7	C. Brodski	NM_013613
cPatched1	NotI	SP6	R. Klafke	NM_204960.1
cPax6	EcoRI	T7	J. Goulding	NM_205066
cPitx3	NotI	T3	BBRSC	XM_421631
mPitx3	NotI	SP6	J. Guimera	NM_008852
cShh	XbaI	SP6	T. Nohno	NM_204821.1
cTh	NotI	T3	BBRSC	NM_204805.1
cWnt1	EcoRI	T7	A. McMahon	AY753286
mWnt1	HindIII	T7	C. Brodski	NM_021279
cWnt3a	NotI	T3	BBRSC	NM_204675
cWnt4	NcoI	SP6	C. Hartmann	NM_204783
cWnt5a	EcoRI	SP6	A. McMahon	NM_204887
cWnt5b	EcoRI	SP6	A. McMahon	NM_001037269
cWnt6	XbaI	SP6	R. Klafke	NM_001007594
cWnt7a	XhoI	T3	A. McMahon	NM_204292
cWnt7b	HindIII	T3	A. McMahon	NM_001037274
cWnt9a	SpeI	T7	C. Hartmann	NM_204981
mWnt9a	SpeI	T7	J. Zhang	NM_139298

Table 1: List of used *in situ* probes. The plasmids were linerarized with the enzymes named in the second row and transcribed with the RNA polymerases named in the third row. EST clones were ordered online from the BBRSC ChickEST database.

7.4 Antibodies

Antigen	Species	Host	Conjugate	Dilution	Company	Cat. No.
GFP		Rabbit	-	1:500	ams	TP-401
BrdU	-	Mouse	-	1:10	Roche	1299964
HuC/D	Human	Mouse	-	1:300	Molecular Probes	A-21271
IgG(H+L)	Mouse	Goat	Biotin-SP	1:500	Jackson	115067003
IgG(H+L)	Rabbit	Goat	Cy2	1:100	Jackson	111097003
IgG(H+L)	Mouse	Goat	Cy3	1:100	Jackson	115167003

Table 2: List of applied antibodies with dilution factors. (H+L) heavy and light chain of the immunoglobulin molecule; HRP horseradish peroxidase; biotin-SP biotin with long spacer (Jackson Immunoresearch); Cy2 Cyanine; Cy3 Indocarbocyanine.

7.5 Abbreviations

∅	diameter
A	absorbance
A/P	anterior-posterior
aa	amino acids
Aldh1a1	retinaldehyde dehydrogenase I
ANR	anterior neural ridge
AP	alar plate
BCIP	5-bromo-4-chloro-3-indonyl-phosphate
bHLH	basic helix-loop-helix
Bmp	Bone morphogenic protein
bp	base pairs
BP	basal plate
BrdU	Bromo-2´-deoxy-uridine
BSA	bovine serum albumine
c	centi (10^{-2})
°C	degrees Celsius
cDNA	copy-DNA
Ci	Curie
CMV	cytomegalovirus
CNS	central nervous system
CO_2	carbon dioxide
cmp	counts per minute
CRD	cysteine rich domain
CRD	cysteine rich domain
F	Farad
Fig.	figure
FP	floor plate
D/V	dorso-ventral
Da	Dalton
DA	dopaminergic
DAB	3,3´-diaminobezidine
dATP	2´-deoxyadenosine-5´-tri-phosphate
dCTP	2´-deoxycytidine-5´-tri-phosphate
dGTP	2´-deoxyguanine-5´-tri-phosphate
di	diencephalon
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DNA	2´-deoxyribonucleic acid
dNTP	2´-deoxynucleoside-5´-tri-phosphate
dTTP	2´-deoxythymidine-5´-tri-phosphate
E	embryonic day
E. coli	*Escherichia coli*
e.g.	for example
EDTA	ethylenediamine tetraacetate
En	Engrailed
ES	embryonic stem
NH_4OAc	ammonium acetate
Nkx	NK transcription factor related

Frz	Frizzled
g	gravity constant
g	gram
GOF	gain-of-function
Gsk	Glycogen synthase kinase
h	hours
hb	hindbrain
HEPES	N-2-hydroxyethylpiperazine-N´-2-ethane sulfonic acid
HLH	helix-loop-helix
HRP	horseradish peroxidase
H_2O_2	hydrogen peroxide
i.e.	that is (latin: *id est*)
INT	2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium chloride
J	Joule
k	kilo (10^3)
kb	kilobases
KCl	potassium chloride
kDa	kilo Dalton
KH_2PO_4	potassium dihydrogen phosphate
l	liter
Lef	Lymphoid enhancer binding factor
Lmx	LIM homeobox transcription factor
LOF	loss-of-function
µ	micro (10^{-6})
m	meter
m	milli (10^{-3})
M	molar
Mash	Mammalian ac haete-scute homolog
Math	Mammalian athonal homolog
mb	midbrain
MES	2-(N-morpholino)ethane sulfonic acid
mes	mesencephalon
mesDA	mesencephalic dopaminergic
met	metencephalon
$MgCl_2$	magnesium chloride
MHB	mid-/hindbrain boundary
min	minutes
MOPS	3-(N-morpholino)propane sulfonic acid
mRNA	messenger RNA
Msx	Msh like
myel	myelencephalon
MZ	mantle zone
n	nano (10^{-9})
NaCl	sodium chloride
Na_2HPO_4	sodium hydrogen phosphate
NBT	4-Nitro-blue tetrazolium chloride
NCBI	National Center for Biotechnology Information
NP-40	Nonidet-P 40
Nr4a2	Nuclear receptor sub-family4, groupA,member 2
NTP	nucleoside-5´-triphosphate
OD	optical density
Otx	Orthodentricle homolog
pA	polyadenylation signal
PAGE	polyacrylamide gelelectro-phoresis
Pax	Paired box gene
PBS	phosphate buffered saline
Pbx	Pre B-cell leukemia transcription factor
PCR	polymerase chain reaction
PFA	paraformaldehyde
Pitx	Pituitary homeodomain transcription factor
PNS	peripheral nervous system
RNA	ribonucleid acid
RP	roof plate
RT	room temperature
RT-PCR	reverse transcriptase PCR
s	seconds
SDS	sodium dodecylsulfate
Shh	Sonic hedgehog
Slc	Solute carrier family
SV	simian virus
SVZ	subventricular zone
Tcf	T-cellspecific transcription factor
tel	telencephalon
Tgf	Transforminggrowth factor
Th	Tyrosine hydroxylase
Tris	tris(hydroxymethyl)amino-methan
tRNA	transfer-RNA
U	unit
UV	ultra violet
V	volt
VZ	ventricular zone
W	Watt
WB	Western blot
Wnt	Wingless-related MMTV integration site
ZLI	zona limitans intrathalamica

7.6 Curriculum vitae

2/2004 – 5/2008

Graduate studies in Developmental Neurobiology, Helmholtz Center Munich, German Research Center for Environmental Health / Technical University Munich

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*both authors contributed equally to this work

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Hiermit versichere ich, dass ich mich zu keiner Zeit anderweitig um die Erlangung des Doktorgrades beworben habe.

Ferner versichere ich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe und keine anderen als die hier vorliegenden Quellen und Hilfsmittel benutzt und die den verwendeten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Neuherberg, den 11.02.2008

Ruth Klafke