Role of Dmrt5 during development of midbrain dopaminergic progenitors

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Thesis presented for PhD degree

I declare that the work described in this Thesis is my own unless otherwise stated.

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Abbreviations

A/P	Anterior/posterior
ANOVA	Analysis Of Variance
bHLH	Basic helix loop helix
cDNA	Complementary DNA
ChEST	Chicken EST
ChIP	Chromatin Immunoprecipitation
CNS	Central nervous system
D/V	Dorsal/ventral
DNA	Deoxyribonucleic acid
EpiSC	Epistem cell
ES	Embryonic stem cells
EST	Expressed sequence tag
FP	Floor plate
IPA	Ingenuity Pathways Analysis
IsO	Isthmus
IZ	Intermediate zone
mRNA	Messenger RNA

MZ	Marginal zone
NS	Neural stem cells
OCM	Ocular Motor
PCA	Principal components analysis
PCR	Polymerase chain reaction
PD	Parkinson's disease
RN	Red Nucleus
RNA	Ribonucleic acid
RrF	Retrorubral field
SNc	Substantia nigra
SNK	Student-Newman-Keuls
VLM	Ventral lateral midbrain
VLMd	VLM electroporated with Dmrt5
VLMg	VLM electroporated with GFP
VLMi	VLM exposed to current alone
VTA	Ventral Tegmental area
VZ	Ventricular zone

Abstract

This thesis investigates the previously un-described role of Dmrt5, a zinc-finger transcription factor, in ventral midbrain development. The spatio-temporal expression pattern of *Dmrt5*, described for the first time in this thesis, suggests that Dmrt5 may have a role in midbrain dopaminergic development.

Exogenous expression of *Dmrt5,* by *in ovo* electroporation in the chick embryo and over expression during neuronal differentiation of mouse epistem cells, causes ectopic expression of *Hes1*, a gene involved in the timing of neurogenesis. *Dmrt5* ectopic expression also reduces the number of cells undergoing proliferation and inhibits terminal differentiation. These findings are consistent with the previously described phenotype observed upon *Hes1* over expression. Studies in neuronal progenitors derived from mouse epistem cells indicate Dmrt5 mediates this effect by binding to the *Hes1* promoter region.

Genome-wide analysis of *Dmrt5* over expression within the developing chick embryo, implicate Dmrt5 in specification of dorsal-ventral patterning. *Dmrt5* promotes a floor-plate-like transcriptional profile when it is ectopically expressed in the dorsal ventral lateral midbrain. This observation is confirmed by ectopic expression studies in the developing chick and by over expression studies in neural progenitors differentiated from mouse epistem cells.

In conclusion the data presented in this thesis describe the role of Dmrt5 in neurogenesis and dorsal-ventral patterning of the ventral midbrain. This data contributes to our knowledge of the developmental program involved in the creation of midbrain dopaminergic progenitors.

Chapter 1. Introduction

1.1 Dopamine neurons

Neurons producing the catecholaminergic neurotransmitter dopamine are present in various areas of the central nervous system (CNS), as illustrated in Figure 1.1. They are typically identified by the presence of tyrosine hydroxylase (TH), the rate limiting enzyme for dopamine biosynthesis.[1, 2]

The largest population of dopaminergic neurons in the brain are found in the ventral midbrain. The majority of these midbrain dopaminergic neurons form the substantia nigra pars compacta (SNc, or A9 group) and the ventral tegmental area (VTA, A10 group), with the remaining midbrain dopamine neurons found in the retrorubral field (RrF, A8). These midbrain dopamine neurons project to the striatum, limbic and cortical areas forming the nigro-striatal, meso-limbic and meso-cortical pathways. (Figure 1.1) [3, 4]

Although the majority of dopamine neurons are found in the midbrain there are populations of dopamine neurons in the diencephalon involved in neuroendocrine control. These diencephalic dopamine neurons, known as the A11-15 groups, include dopamine neurons of the posterior hypothalamus (A11) which project to the autonomic areas of the lower brain stem and spinal cord [5, 6], and the zona incerta in the ventral thalamus (A13) which project diffusely to different areas of the hypothalamus and to the amygdala [7, 8]. There is also a small population of dopamine neurons in the telencephalon, the A16 group of the olfactory blub periglomerular interneurons that serve to modulate sensory activation in the olfactory

bulb [9-11]and the A17 group of retina amacrine interneurons involved in adaptation to light and dark [12, 13]. (Figure 1.1)

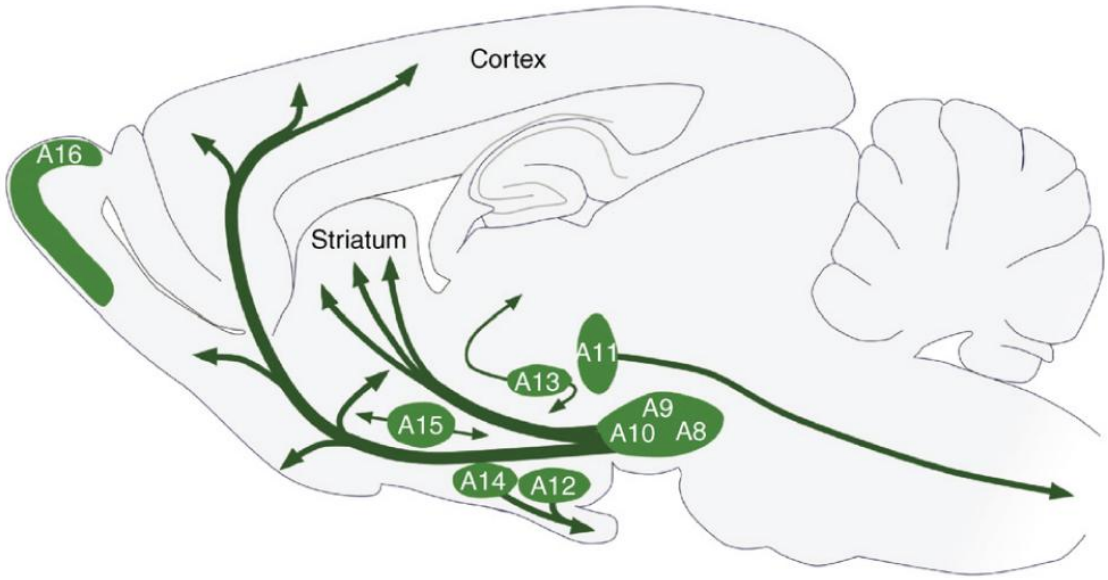


Figure 1.1. Location of dopamine neurons cell groups in the adult rodent brain. Distribution of dopamine neuron cell groups in the adult brain. The numbering of these cell groups was introduced by Dahlström and Fuxe in 1964 [2]. Image is taken from Björklund and Dunnett [14].

There is much interest in the midbrain dopaminergic neurons of the SNc and VTA, which function in motor control, reward and emotive behaviour, as their dysfunction and degeneration is involved in several diseases [15]. Preferential degeneration of SNc neurons projecting to the striatum leads to the characteristic symptoms of Parkinson's disease (PD), namely hypokinesia resting tremor and rigidity [16-18]. This disease is one of the most common neurodegenerative diseases, affecting about 2% of the population over 65 years [19, 20]. The cause of the neurodegeneration seen in PD patients is largely unknown, with the majority of the

cases being sporadic [21, 22]. To date (2011), more than 10 genes and several additional genetic loci have been associated with familial forms of the disease [22-29]. These indicate that abnormally increased oxidative stress and mitochondrial dysfunction, together with protein misfolding and impairments in the ubiquitin–proteasome and autophagy–lysosomal systems contribute to PD [19, 30, 31].

PD patients are currently treated with a variety of pharmacological tools, including L-DOPA, dopaminergic agonists and inhibitors of dopamine degrading enzymes [17, 19, 32]. The current surgical treatment of PD is mainly based on the use of deep brain stimulation of the internal segment of the globus pallidus, the subthalamic nucleus or the pedunculopontine nucleus [33]. While these treatments provide symptomatic relief, none of them change the course of the disease [34]. There is therefore a clear need for restorative and regenerative approaches.

The discovery in the rat that foetal dopamine neurons from the ventral midbrain can be transplanted into 6-hydroxydopamine lessioned dorsal striatum and restore impaired motor functions [35] led to the experimental use of dopamine grafts in individuals with Parkinson's disease as a possible restorative method [36-41] [42]. In clinical trials dopamine-producing human foetal midbrain tissue was transplanted into the striatum of PD patients. Patients in some trials have shown increased numbers of dopamine neurons within the nigro-striatal pathway and improvements in general motor performances [36-41], however the results are inconsistent [43-46]. Despite their inconsistencies, these trials provide proof of principle that replacement therapy may provide a prophylactic treatment for PD. However use of foetal tissue for transplantation, as a routine treatment, is both impractical and unethical. Meanwhile, grafting of embryonic stem (ES) [47-52] and neural stem (NS) cells [52-54] into the striatum of animals with PD characteristics to improve motor symptoms has proved

unsuccessful. This highlights the need to use cells which already exhibit midbrain dopaminergic identity [55-57]. As a result there is great interest in creating midbrain dopaminergic neuronal progenitors from pluripotent cells *in vitro*.

In addition to degeneration of the nigro-striatal projection causing PD, dysregulation of dopamine synaptic transmission in the meso-limbic system has also been linked to the development of drug addiction [58-60] and depression [61]. Exaggerated forebrain (meso-cortico-limbic projections) dopamine transmission is thought to contribute to psychotic symptoms of schizophrenia [62, 63], which has a worldwide incidence of 15 per 100,000 [64]. Addiction, depression, neurodegenerative diseases and schizophrenia, all major public health problems of the industrialised world, are linked to degeneration or dysfunction of the ventral midbrain dopaminergic neurons of the SNc and VTA. Therefore understanding the behaviour of these neurons is of major importance.

The non-proliferative nature of neurons and the inaccessibility of this population mean further study of midbrain dopamine neurons requires generation of these neurons *in vitro*. These created neurons would be useful for a number of applications, such as: pharmacological assays to analyse the activity of potential drug treatments for midbrain dopaminergic system disorders, comparative studies of diseased neurons derived from patient cells and healthy neurons to identify possible mechanisms and causes of the disease. Finally, as studies in animal models have indicated that transplantation of dopaminergic neurons into the SNc can alleviate the motor symptoms of PD [35, 36]; the generation of these neurons from stem cells or induced pluripotent patient cells could provide a potential therapy for this increasingly prevalent disease.

To create functional midbrain dopaminergic neurons, which resemble *in vivo* counterparts, from pluripotent cells *in vitro*, it is thought that a cascade of transcription resembling normal *in vivo* development must be triggered. Current *in vitro* differentiation methods mimic the known *in vivo* developmental pathways, but still give low yields of dopaminergic neurons with many not displaying an authentic dopaminergic profile [65-67]. To improve *in vitro* generation of dopamine neurons a better understanding of midbrain dopaminergic neuron specification and development is required. Our current understanding of midbrain dopaminergic neuronal development is discussed below, followed by a summary of *in vitro* generation methods currently used.

1.2 Development of midbrain dopaminergic neurons

Dopaminergic neurons arise in the ventral midbrain. Formation of this region is mediated by two signalling centres, the isthmus (IsO) which determines the Anterior/Posterior (A/P) axis of the midbrain, and the floor plate which defines the Dorsal/Ventral (D/V) axis. These signalling centres potentiate cells located in the medial ventral midbrain to dopaminergic fate by triggering expression of specific transcription factors. Following specification, differentiation of these progenitors results in the formation of mature midbrain dopaminergic neurons. In the following sections I will discuss these 3 steps.

1) Formation of the ventral midbrain and potentiation of dopaminergic neurons

2) Specification to dopaminergic fate

3) Terminal differentiation

1.2.1 Formation of the midbrain by A/P and D/V patterning leads to potentiation of the ventral midbrain to dopaminergic fate

After its formation, the neural plate is sub-divided into restricted domains. This results in formation of the midbrain, forebrain, hindbrain, and spinal cord regions of the neural epithelium. With regard to formation of the midbrain, this is determined by formation and positioning of the IsO at the midbrain-hindbrain border and the floor plate at the ventral midline, both of which express signalling factors required for midbrain dopaminergic neuron development [68, 69].

1.2.1.1 Formation of the isthmus

The isthmus (IsO) locates the junction between the midbrain and hindbrain, it is necessary and sufficient for development of the midbrain and hindbrain structures [70-72]. Transplantation of the tissue encompassing the IsO to another region of the neural tube can induce midbrain and hindbrain cell fate in host territories [71-75]. Conversely, if neural tissue, such as the prosencephalic tissue, is grafted into the IsO this acquires midbrain and hindbrain cell fates [76, 77], while removing the IsO triggers loss of midbrain and hindbrain structures [78]. The IsO controls the rostro-caudal specification of the midbrain and hindbrain as grafting and transplantation experiments showed that the induction of midbrain or hindbrain is always polarised [71-73, 75-77].

At the onset of somitogenesis paired box gene 2 (Pax2) and wingless-type MMTV integration site family member 1 (Wnt1) are expressed in broad and overlapping domains within the IsO [79], with Wnt1 restricted to the midbrain side and Pax2 [80] followed by En2 (at 3-5 somite stage) and paired box gene 5 (Pax 5) (3-5 somite

stage) expressed across the isthmus border [81-83]. The secreted factor, fibroblast growth factor 8 (FGF8), is expressed at the 3-5 somite stage, but is restricted to the caudal hindbrain [80]. The boundaries between the midbrain and hindbrain are subsequently refined to form the IsO. At this point Wnt1 expression is down regulated such that it is now only present in a ring of cells in the neural tube just rostral to the IsO [84-86]. These observations regarding the formation of the boundary between the midbrain and hindbrain indicate that this IsO is roughly positioned during late gastrulation and then is progressively refined during early somitogenesis. Complete or partial loss of Wnt1 and FGF8, which are expressed on either side of the IsO, leads to a gradual loss of midbrain and hindbrain, indicating these genes control the maintenance of the IsO [86-92].

1.2.1.2 Floor plate provides dorsal ventral patterning

Formation of the ventral midline occurs when the neural plate undergoes neurulation. During this process the most lateral regions of the neural tissue of the neural plate are elevated, this leads to bending of the neural plate and formation of a neural groove. The medial movement of the neural folds towards the midline causes the two sides of this neural tissue to meet and fuse, sealing the neural tube [93, 94]. The notochord lies directly under the midline and expresses Sonic Hedgehog (Shh); this expression induces Shh expression in the floor plate [95-102]

1.2.2 Potentiation of ventral midbrain

Midbrain dopaminergic neurons arise from the Shh expressing region of the floor plate, rostral to the IsO [103-107]. Indeed co-culture of explant of E9 rat floor plate with dorsal midbrain can induce ectopic dopaminergic neurons [95]. The inductive

effects of the floor plate can be mimicked by graded doses of Shh [69, 95, 99, 108-110], and its effects are prevented by Shh-blocking antibodies [69], suggesting that Shh is responsible for the observed floor plate activity and that this is involved in formation of midbrain dopaminergic neurons.

Whilst Shh is expressed within the floor plate of the entire neural tube, dopaminergic neurons are only found in the midbrain, suggesting that Shh alone is not sufficient for dopamine neuron generation [103, 105, 106]. Moreover, exposure to exogenous Shh cannot induce ectopic dopaminergic neurons in the hindbrain, ventro or dorsocaudal forebrain [69]. In a similar pattern, loss of FGF8 from E9 midbrain floor plate explants prevents formation of dopaminergic neurons, despite normal Shh expression [69]. However, the IsO or FGF8 alone are not sufficient to induce dopamine neurons in the absence of Shh [69]. Rather the integration of anterior-posterior and dorsal-ventral patterning information of the IsO and floor plate respectively are required in order to create dopamine neurons [69]. This FGF8 and Shh co-expressing region which gives rise to midbrain dopaminergic neurons is shown in Figure 1.2. In support of this hypothesis, ES cells exposed to FGF8 and Shh differentiate to midbrain dopaminergic fate [65, 111, 112]. It is important to note that whilst this potentiation process enables cells to become dopaminergic it does not commit them solely to this fate.

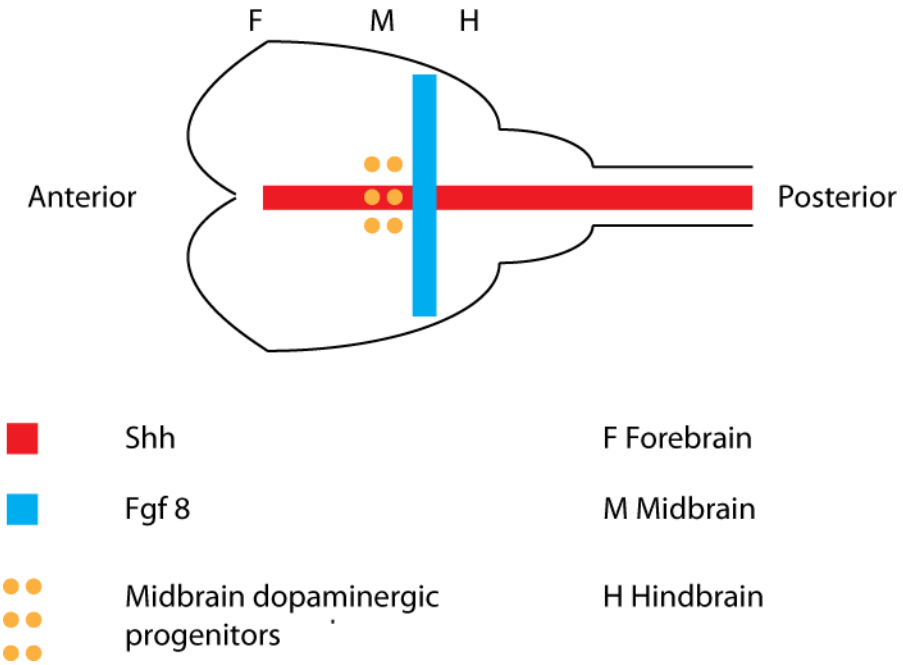


Figure 1.2. Schematic representation of potentiation of midbrain dopaminergic neurons in a horizontal plane. Co-ordinate signalling of FGF8 from the isthmus (blue) and Shh signalling from Floor plate (red) induce midbrain dopaminergic progenitors in the ventral midbrain (orange). Image represents mouse E8.0 or chick HH st 10.

The signalling from the IsO and floor plate convey specific neuronal identity to the ventral midbrain cells by triggering the expression of specific transcription factors which specify dopaminergic fate to the most medial population, these transcription factors will be discussed in the next section. Figure 1.3 depicts the developing brain, highlighting the region which gives rise to midbrain dopaminergic neurons.

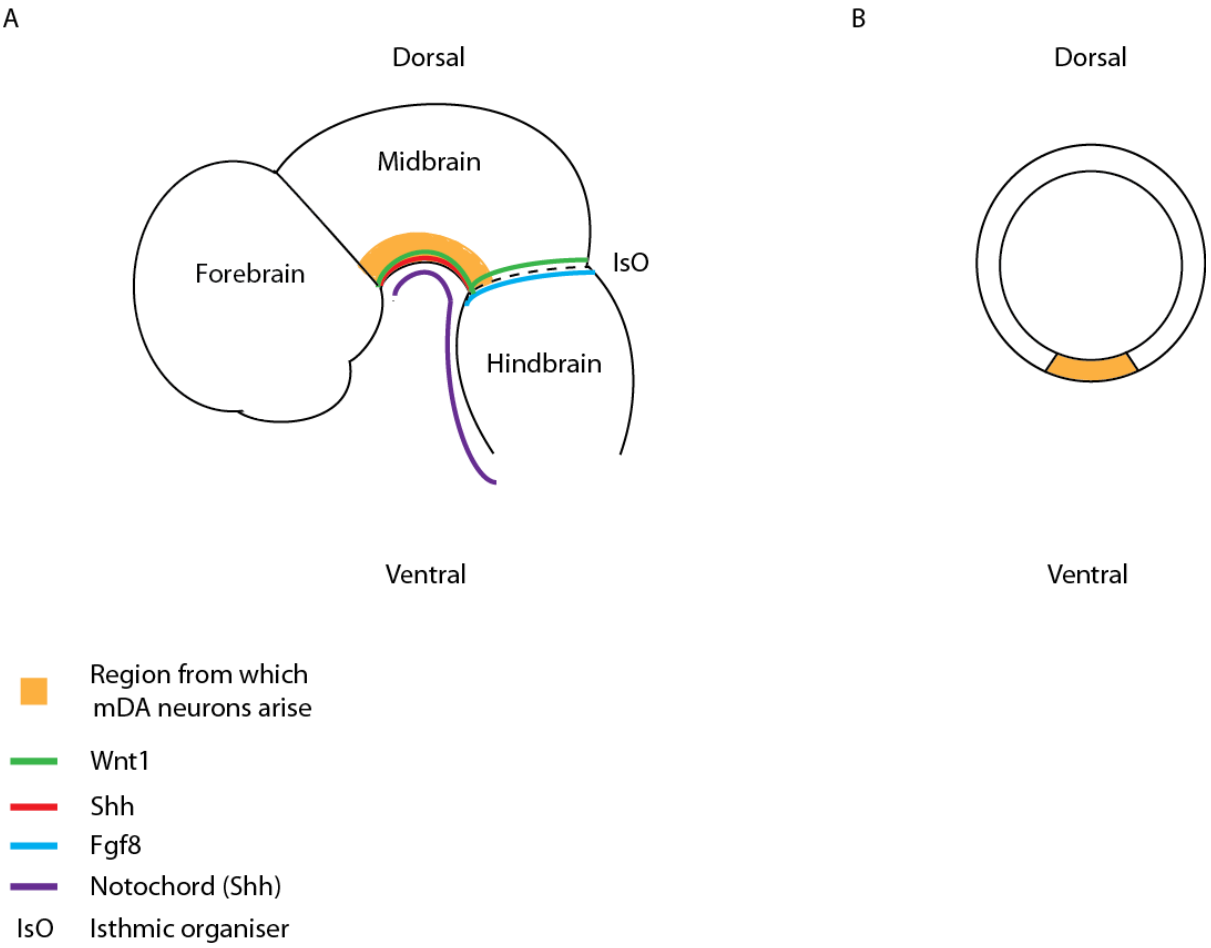


Figure 1.3. Schematic view of developing brain. Midbrain dopaminergic neurons arise from the region in orange due to signalling of FGF8 from the isthmus (blue), Wnt1 signalling (green) and Shh signalling from the floor plate (red) and notochord (purple). (B) Coronal section through the midbrain, highlighting, in orange, the region from which midbrain dopaminergic neurons arise.

1.2.3 Specification of neurons to dopaminergic fate

Specification commits potentiated cells to midbrain dopaminergic fate and occurs in the ventricular zone from around E9.5 onwards (Figure 1.3). The known key players in this process are the transcription factors LIM homeobox transcription factor 1 A/B **(**Lmx1A/B), Forkhead box A1/2 (FoxA1/2), Neurogenin2 (Ngn2), Msh homeobox 1/2 **(**Msx1/2), and Orthodenticle homeobox 2 (Otx2) [103, 105, 113-119] whose

expressions are triggered by the signalling of Wnt1 and FGF8 from the IsO and Shh from the FP [69, 107, 120-122].

In order to commit the cells to a dopaminergic phenotype, the midbrain dopaminergic specification genes block expression of genes involved in the specification and neurogenesis of the more lateral midbrain fate, such as the immediately adjacent Red Nucleus (RN) by inhibiting expression of genes such as Brna3, Sim and Nkx2.2 and Nkx6.1, and GABAergic fate by inhibition of Helt [123-125]. As well as blocking lateral fates, specification of dopaminergic neurons by Lmx1A/B, FoxA1/2, Otx2, Msx1/2, and probably other factors, triggers neurogenesis, which is characterised by expression of Ngn2 [114, 117].

Neurogenesis within the ventral midbrain occurs from around E10 to E14 in mice with the highest rate at E11.5 [126]. Generation of midbrain dopamine neurons requires the proneural gene Ngn2, which is expressed in the ventricular and intermediate zone [114, 117]. Mash1, which is also expressed at a lower level in the ventricular zone, is not required, but can partially compensate for loss of Ngn2 [117]. Concomitant with neurogenesis, exit from the cell cycle occurs [127] during this process cells move away from the ventricular zone to form the intermediate zone [114, 117]. Expression of proneural genes and proliferation seem to be linked as loss of Ngn2 prevents cells in the ventricular zone from undergoing cell cycle exit [114, 117].

Thus specification ensures the floor plate region has the correct transcriptional profile to create midbrain dopaminergic neurons and to undergo differentiation into mature neurons co-expressing TH and Paired homeobox protein 3 (Pitx3).

A working model of the gene networks involved in specification can be seen in Figure 1.4. Floor plate expression of Shh activates expression of FoxA2 via Gli2 binding sites on the FoxA2 promoter [120]. FoxA2 then directly activates Shh expression leading to a positive feedback loop [128]. FoxA2 is also required for the initiation of FoxA1 at E9.5 [125], and conditional loss of FoxA1/2 in neural progenitors reduces Lmx1A/B expression [124]. Wnt1 expression from the IsO, which signals via the canonical pathway, activates transcription of Lmx1A, Lmx1B and Otx2 via β-catenin TCF/LEF sites on the promoter of these genes [121]. It is also possible that other canonical Wnts expressed in this region, namely Wnt2 and Wnt3A could also be involved in this process [129, 130]. Furthermore Lmx1A directly activates transcription of Wnt1 forming a regulatory loop between these two genes [121].

Following activation of Lmx1A/B, FoxA1/2 and Otx2, expression of further transcription factors involved in specification, namely Msx1 directly activated by Lmx1A [115, 121] and Ngn2 activated indirectly via Lmx1A , Otx2, Msx1 and FoxA2 occurs [115, 118, 119, 131]. At the same time expression of the genes required for terminal differentiation, > and Pitx3 are initiated directly by Lmx1A/B [121] and by an unknown FoxA2 mediated mechanism [118, 124].

The only known gene sufficient for specification to dopaminergic fate is Lmx1A, as ectopic expression of this gene leads to induction of midbrain dopaminergic neurons in ES cells and in electroporated chick ventral midbrain [115]. However, Lmx1A only has an effect on cells in the ventral midbrain, cells that have been potentiated [115]. This is postulated to be because it regulates some targets cooperatively with FoxA2 and is thus only effective in FoxA2 positive regions [124].

Specifically co expression of Lmx1A and FoxA2 is required for the inhibition of Ngn1 in the ventral midbrain [123]. Ngn1 is expressed in the more lateral RN [132] and inhibits midbrain dopamine fate by repressing Pitx3 [123]. The co-expression of FoxA2 and Lmx1A is also required for the inhibition of Shh, the down regulation of which may be required to induce neurogenesis in the ventral midbrain at later stages of specification. While FoxA2 positively regulates Shh at early stages [120], at later stages of specification, Foxa1/2 regulates ventral midbrain patterning by decreasing expression of intracellular transducers and downstream targets of Shh in the ventral midbrain [125]. Inhibition of this process prevents development of midbrain dopaminergic neurons [125].

Lmx1A alone can indirectly induce Ngn2 which is specifically required to initiate neurogenesis towards dopaminergic fate [123]. This is in part by Lmx1A mediated repression of Helt (an Ngn2 repressor) normally expressed in more lateral regions and which gives rise to GABAergic neurons [123, 132, 133]. Lmx1A has also previously been reported to up regulate Ngn2 via directly activating Msx1, which inhibits expression of Nkx6.1 [115] another gene found in the adjacent RN region. However, the precise role of the repression of Nkx6.1 by Msx1 is not fully understood, as over expression of Nkx6.1 does not prevent midbrain dopaminergic differentiation [123].

In keeping with the role of Lmx1A in repressing the RN fate, Lmx1A also represses the FoxA2 induced Sim expression and thereby prevents expression of Lhx1, a gene which inhibits differentiation to dopaminergic fate [123]. This indicates that FoxA2 induces differentiation programs for midbrain dopaminergic neurons but also induces a lateral RN fate too. Lmx1A in the ventral midbrain selects the dopaminergic fate by suppressing the RN fate. These findings highlight that Lmx1A functions to promote

neurogenesis via regulation of Ngn2 and influences patterning [115] to ensure the specification of midbrain dopaminergic neurons.

Lmx1B and 1A are expressed in the same domains and have a 61% conservation of amino acid identity [134]. Recent chromatin Immunoprecipitation assays of both ES cells and embryonic tissues have shown that like Lmx1A, Lmx1B can bind to the promoters of Nuclear hormone receptor 1 (Nurr1), Msx1 and Pitx3 [121]. This may explain why knock out of Lmx1A has mild abnormalities in midbrain dopaminergic neuron development [123]. While Lmx1B and A seem to regulate many of the same target genes, loss of Lmx1B leads to TH cells with no Pitx3 expression at E12.5 and an ectopic population of cells that express Pitx3 but not TH [135]. This indicates that Lmx1B is required for proper differentiation of midbrain dopaminergic neurons in a way which cannot be rescued by Lmx1A. In regard to this, it is important to consider that Lmx1B is expressed prior to Lmx1A. The early expression of Lmx1B is involved in potentiation of the medial ventral midbrain, therefore in Lmx1B knock out mice this role in dopaminergic neuron development could not be compensated for by Lmx1A [136, 137].

Otx2 like Msx1 and Lmx1A can repress expression of genes involved in more lateral phenotypes such as the Nkx6.1 and Nkx2.2 genes, expression of which is initiated by FoxA2 and Shh. Otx2 repression of these genes ensures differentiation to dopaminergic fate, as Nkx2.2 is important in inducing the serotonergic neuronal lineage in hindbrain and act as a suppressor of dopaminergic fate [122, 138]. Otx2 is integral to midbrain dopaminergic neuron development as conditional loss of Otx2 during specification results in a severe reduction in the expression of Lmx1A, Msx1, and Ngn2 within the ventral midbrain and these cells fail to express Nurr1, proliferation defects are also observed [139].

Concomitant with expression of Ngn2 and repression of lateral fates is the expression of Nurr1 and Pitx3 which are required for development of the dopamine signalling machinery such as transporters and synthesis enzymes [140]. Chromatin Immunoprecipitation shows that Lmx1A and 1B can directly activate Pitx3 and Nurr1, whilst FoxA2 when expressed in combination with Lmx1A also seems to be involved in Nurr1 activation. This leads to the initiation of the third step in development of midbrain dopaminergic neurons, terminal differentiation.

1.2.4 Terminal differentiation of dopaminergic neurons

Terminal differentiation triggers the expression of all enzymes, transporters and receptors required for proper synthesis, storage, release and reuptake of dopamine. This includes expression of TH, the rate limiting step for dopamine synthesis, aromatic L-amino acid decarboxylase (AADC) needed for the final enzyme in the synthesis of dopamine, Vesicular monoamine transporter 2 (VMAT2) involved in dopamine storage, and Dopamine Transporter (DAT) involved in dopamine reuptake [141].

Conventionally, dopaminergic neurons are considered terminally differentiated when they express TH. The first TH positive dopaminergic neurons are seen at E10.5 [142]. However, most terminal differentiation occurs from E12.5 onwards [143]. During terminal differentiation the neurons leave the intermediate zone to form the marginal zone, from where they migrate to their target tissue. (Figure 1.4)

In vivo conditional knock out of factors involved in dopamine synthesis and transport in dopamine neurons causes no change in the midbrain neuronal development or projections, but prevents these neurons from transmitting dopamine to their target

tissues correctly [144-146]. This highlights the fact these genes are solely required for the functioning of the neurons. Terminal differentiation is mediated by the transcription factors Nurr1, Pitx3, and FoxA2, which are initiated during specification [118, 141, 147-150].

Nurr1 is required for expression of TH, Vmat2, Ret and DAT, as expression of these genes is lost in the Nurr1 null embryo [147, 149-152]. The Nurr1 pathway appears to be distinct from specification as Nurr1 null mice have a correctly specified midbrain dopaminergic neural lineage expressing Pitx3 and Aldh1a [153]. Nurr1 directly regulates TH expression via activating the NGF1 responsive element in the TH promoter region after its own phosphorylation by Erk1/2. [154]. Nurr1 appears to have an additional role as these neurons fail to migrate laterally to their normal positions during development and were unable to innervate their target tissue [153].

Paired-like homeodomain transcription factor 3 (Pitx3) is expressed in midbrain dopaminergic neurons in the marginal zone from E11 onwards [155-157], null mice show preferential loss of SNc neurons while VTA neurons are affected to a lesser extent [158, 159]. Prior to cell death the down regulation of TH in the SNc population was observed [159]. As both VTA and SNc express Pitx3 these findings suggests that VTA and SNc neurons differ in their molecular make up. The regulation of TH via Pitx3 also differs in these populations of neurons, with SNc neurons expressing Pitx3 prior to TH, whilst VTA neurons express TH and Pitx3 simultaneously [159]. Pitx3 has been shown to bind to the promoter of TH indicating Pitx3 may directly regulate TH *in vitro* [160-162]. However over expression of Pitx3 in hippocampus progenitors didn't cause an increase in TH [163] suggesting that Pitx3 can only have an effect in specific cell types, unlike Nurr1 which seems to activate TH expression independent of cellular context [164]. Pitx3 null mice are also deficient in Aldh1a and

DAT [165]. Aldh1a, a retinoic acid synthesising enzyme, is a SNc specific Pitx3 target gene and can partially rescue Pitx3 deficient phenotype [166, 167] suggesting that Aldh1a acts downstream of Pitx3 in the SNc neurons.

While both Pitx3 and Nurr1 regulate expression of the known genes involved in terminal differentiation, over expression of either of these transcription factors alone promotes expression of only a subset of terminal differentiation markers [66, 163, 164, 167-170]. Co-expression of both Pitx3 and Nurr1 during neuronal differentiation of ES cells induces the highest expression of TH, VMAT, DAT and Tuj1 [170]. The authors therefore propose that Nurr1 and Pitx3 act synergistically to induce the terminal differentiation of midbrain dopaminergic neurons [170].

As well as triggering expression of dopamine transmission and synthesis machinery, terminally differentiated and differentiating neurons require factors for survival. En1/2 are required for survival of dopaminergic neurons in the late stages of foetal development and in adults [171-174]. In En1/2 null mice all midbrain dopaminergic neurons die between E12, the onset of En expression, and E14 [171]. The En1+/- En2-/- mutant has normal midbrain dopaminergic composition at birth but loses TH neurons in the SNc whilst retaining those of the VTA [173]. This highlights the sensitivity of the SNc neurons which have distinct survival requirements. Understanding the developmental cues required for development of this subset of neurons is essential for therapeutic approaches for PD and may also improve understanding of why the SNc neurons are more vulnerable and preferentially lost in PD. In addition to Engrailed genes it is thought that retinoid x receptor–Nurr1 heterodimers are involved in survival of mature midbrain dopamine neurons [175].

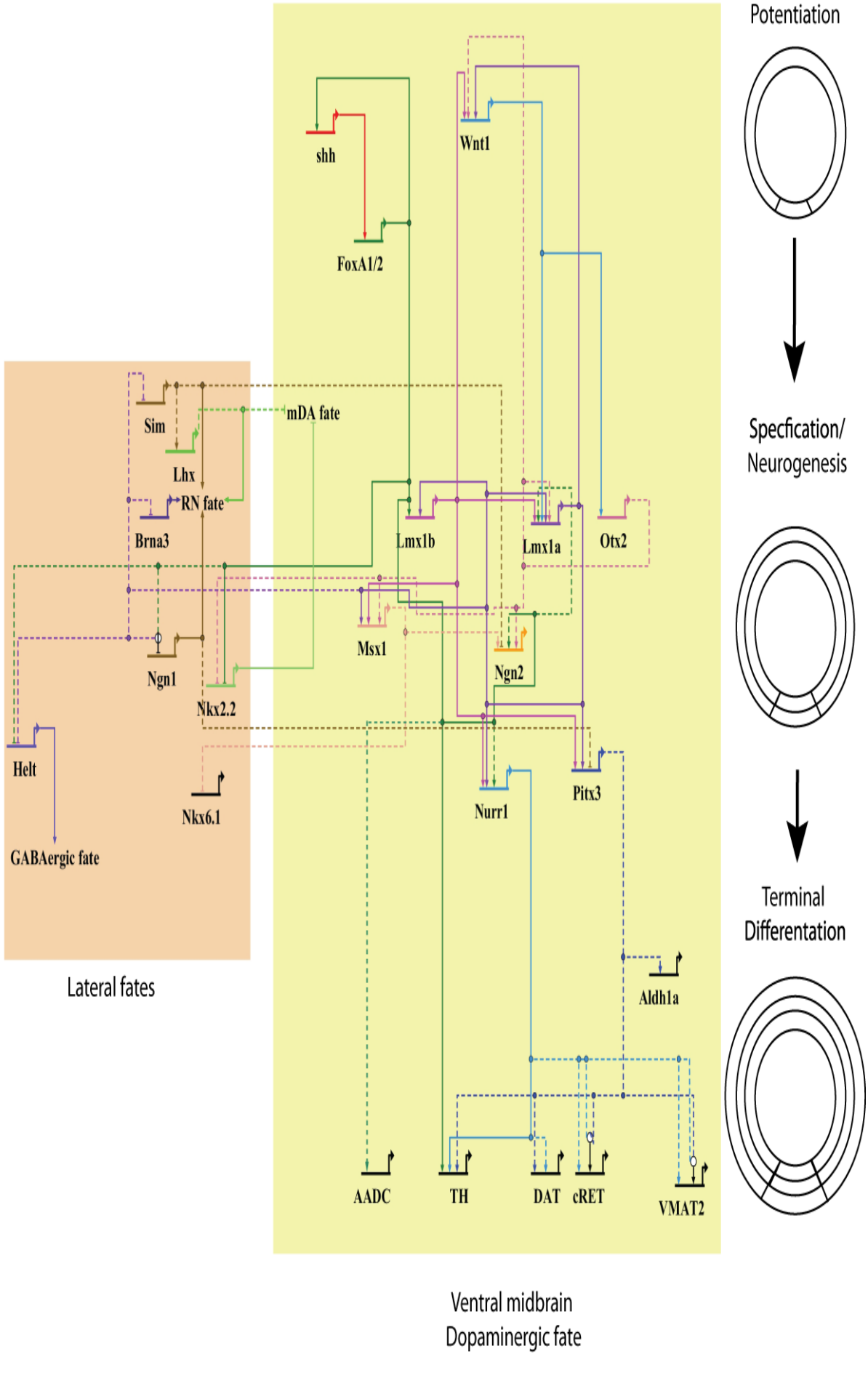

Potentiation
Specfication/
Neurogenesis
Terminal
Differentation
shh
Wnt1
FoxA1/2
Sim
mDA fate
Lhx
RN fate
Brna3
Lmx1b
Lmx1a
Otx2
Msx1
Ngn2
Ngn1
Nkx2.2
Helt
Nkx6.1
Nurr1
Pitx3
GABAergic fate
Aldh1a
AADC
TH
DAT
cRET
VMAT2
Lateral fates
Ventral midbrain
Dopaminergic fate

Figure 1.4. Putative genetic network involved in ventral midbrain differentiation. Solid lines indicate regulation that is direct. To qualify as direct regulation, at least one publication has show by Chromatin Immunoprecipitation binding of the specific protein to the promoter. Dotted lines are regulations which have not been shown to be direct cis-regulation. The information in this network highlights the main players and is inclusive of all published data rather than selective. As several genes, such as FoxA2, are involved at several stages of this process, the gene is placed at the level where expression first starts, i.e. in the potentiation section, however arrows go all the way down to terminal differentiation stage to indicate that this gene continues to be expressed in the third layer and has a role in this later stage as well. Information was taken from the following publications to create this network. [103, 105-107, 114, 115, 117-125, 129, 131, 132, 139, 176-178].

1.3 *In vitro* midbrain dopaminergic specification

In vitro specification methods are based on information obtained from *in vivo* studies. Therefore almost all stem cell based dopamine differentiation protocols start by addition of Shh and FGF8 which potentiate cells to midbrain dopaminergic fate [65, 66, 111, 112, 179]. This typically results in 30% of the neuronal population expressing Tyrosine Hydroxylase (TH) the rate limiting enzyme for dopamine production [180]. However, unless combined with genetic manipulation of midbrain dopaminergic transcription factors such as Pitx3, Nurr1 or Lmx1A [66, 115, 159, 170, 181], the number of neurons with midbrain regional identity generated is low. Furthermore, the yield of TH^+ neurons is inconsistent between experiments and highly variable even within the same culture.

Another protocol which is even less effective for the generation of TH positive cells involves induction of Stromal cell-derived inducing activity (SIDA) [182]. Using this

method, only 15% of SIDA ES cells with TH and Dopamine Transporter (DAT) expression are also Pitx3 positive [183]. Whilst *in vivo* all midbrain dopaminergic neurons simultaneously express these three markers. In summary, current ES cell dopaminergic differentiation protocols produce heterogeneous somatic cell populations and of the dopaminergic neuronal cells within the population not all exhibit true midbrain identity.

One of the major limiting factors in efficient generation of midbrain dopaminergic neurons from stem cells is the heterogeneity of ES cell-derived neural progenitors. Within a given differentiation culture derived from ES cells, progenitors of different developmental stages and/or positional identities are present. As a result of these differences the specific cells within this mixed cell population respond differently to the inductive factors added.

To improve differentiation methods attempts were made in the Li lab to create a more homogenous starting population for *in vitro* differentiation to dopaminergic fate. Epi Stem Cells (EpiSC) are a population of cells which can be obtained from the primitive ectoderm/epiblast stage and as such offer a pluripotent but more restricted and synchronised population of cells [184-186]. Within our lab we have found that EpiSCs convert to neuroectoderm cells more quickly than ESCs when subjected to monolayer differentiation. However without further manipulation the total numbers of $TH^+/Pitx3^+$ cells are still low.

Further research in the lab based on *in vivo* experiments identified that inhibition of FGF/ERK signalling at the start of neural induction during the monolayer differentiation protocol resulted in nearly 40% of TH^+ neurons co-expressing Pitx3 [187]. This improvement may be due to the more synchronous conversion of EpiSC

to neuroepithelial fate which allows efficient stimulation of midbrain dopaminergic competent progenitors, coupled to the early induction of both Lmx1A and FoxA2 and repression of forebrain and hindbrain fates by inhibiting FGF receptor (PD173074) or ERK (PD0325901) [187]. These findings are consistent with the developmental role of FGF signalling in regionalization of the forebrain [188, 189].

This method is based on addition of a small molecule rather than transgene manipulation of Lmx1A, Nurr1 or Pitx3. Therefore it provides an approach which is more easily applicable to generation of midbrain dopaminergic neurons for transplantation and for disease modelling. As this method was the most efficient for generation of midbrain dopaminergic neurons in culture, the use of EpiSCs with the addition of PD173074, at the point of neural induction, was used in cell culture experiments carried out in this thesis.

1.4 Dmrt5

Genes related to the Drosophila melanogaster *Doublesex (dsx)* and Caenorhabditis elegans *Mab-3* are an evolutionary conserved family of transcription factors known as the *Dmrt (Doublesex and Mab-3 related transcription factor)* gene family [190, 191]. Dmrt family members, are characterised by a DNA binding motif known as the DM domain [192], which was first identified in the Drosophila gene *dsx* [193] and later in the C.elegans gene *Mab-3* [192]. All higher metazoan species investigated have multiple DM domain proteins: there are 4 in Drosophila, 11 in C.elegans and 7 in mouse and 8 in humans [191, 194-198]. Outside of the DM domain members of the Dmrt family share little sequence similarity even within species. Despite this the

role of DM family in regulation of sexual differentiation is well conserved across phyla [199, 200].

The DM domain is notable not only for its conserved role in regulating sexual differentiation, but also for to its unusual structure and mode of action [193, 201]. The DM domain is bipartite, comprised of an amino-terminal zinc binding module and a more variable carboxy-terminal domain [202, 203]. The structure of the DM domain is unrelated to the structure of other zinc finger motifs, consisting of an intertwined module coordinating 2 zinc ions [203]. Moreover base substitution experiments indicate the DM domain also differs from other zinc fingers in its interaction with DNA on the minor, rather than major, groove [204]. Mutational analysis indicates that the DM domain interacts with DNA via the zinc binding module and the C-terminal tail mediates high affinity DNA recognition by widening the minor groove without bending the DNA [205].

Binding of Dmrt family members to the minor groove is postulated to enable simultaneous binding of major groove factors to the same region of the enhancer element [206]. The best example of this comes from the enhancer of the yolk protein, *Yp1*, in Drosophila, which contains a binding site for a bZip protein and a Dsx binding site. DSX-F cooperates with the bZip protein to activate *Yp1* transcription in females, while DSX-M is thought to antagonise this protein, helping to ensure that *Yp1* is not transcribed in males [206]. How this occurs is not known, however DSX-M has a larger C-terminal domain than DSX-F which may occlude the binding site of the bZip protein. Furthermore, in C.elegans, Mab-3 binding to the *Ref-1* promoter is thought to repress transcription in part via antagonising activation by an unknown protein that binds to an overlapping site [207].

In vertebrates and C.elegans, Dmrt genes are thought to bind as dimers to a pseudopalindromic DNA site [205]. In support of this, mouse Dmrt3 and mouse Dmrt5 *in vitro* efficiently formed protein DNA complexes that could be supershifted [208]. Mouse Dmrt5 is thought to be a transcriptional repressor, as experiments carried out in the Li laboratory by Xinsheng Nan show that fusion of a Gal-4 DNA binding domain to Dmrt5 represses expression of a UAS Luciferase reporter. An *in vitro* random oligonucleotide selection method to determine the preferred DNA binding sites of 6 of the 7 mouse Dmrt proteins (Dmrt 1-5 and Dmrt7) found that all these protein select very similar DNA sequences despite differences in the primary structure of their DM domains [208]. This indicates that the target specificity of the vertebrate Dmrt family is unlikely to be derived exclusively from the innate DNA binding specificity. In some cases Dmrt heterodimers bound more efficiently than homodimers indicating that heterodimer formation may contribute to transcriptional regulation of Dmrt proteins [209]. Like Dsx, mouse Dmrt5 proteins were found to bind to DNA with high specificity relative to other minor groove proteins [209, 210].

Dmrt5 is a member of the Dmrt A group and as such, along with the DM domain, also contains a DMA motif in its C-terminal, which is also found in Dmrt3 and 4, the function of this conserved region is unknown [198, 211, 212]. Dmrt5 also contains a DMB domain located in the C terminus which is conserved only in the Dmrt5 genes of fish and mammals [213] (Figure 1.5). While no function for Dmrt5 has thus far been described, the role of DM family members in sex differentiation and development has been well described in organisms as phylogenetically divergent as, C.elegans, Drosophila, Xenopus, fish, birds and mammals [211, 213, 214]. This is unusual as most sex specific developmental regulators appear to be conserved only in relatively restricted groups of animals. In addition to a role in sex differentiation

and development, Dmrt4 in Xenopus regulates neurogenesis of the olfactory bulb [215], and Dmrt2 is a regulator of vertebrate segmentation [216-218] demonstrating that the Dmrt proteins also function elsewhere during development. Furthermore the expression patterns of Dmrt genes are not restricted to the gonadal tissues [219]. The known expression patterns of Dmrt family members will be described in the introduction to Chapter 3 which investigates the expression of *Dmrt5* in the chicken embryo.

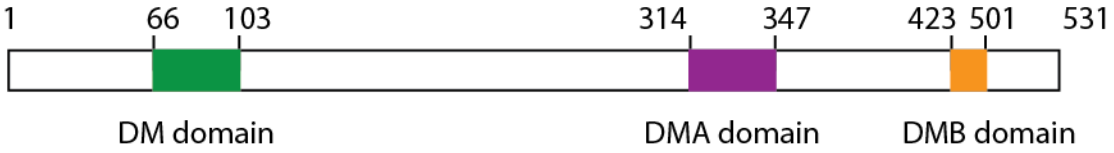


Figure 1.5. Schematic presentation of mouse Dmrt5 protein. Conserved DM, DMA and DMB domains are indicated. Numbers correspond to amino acid positions. Conserved domains were mapped using NCBI conserved domain databank [220].

1.5 Hypothesis and Thesis Aims

The Li Laboratory wish to understand midbrain dopaminergic development further and improve *in vitro* generation of these neurons. Therefore a microarray screen to identify factors expressed in midbrain dopaminergic progenitors of E12 mice was carried out. The screen identified *Drosophila doublesex and C elegans Mab-3 related gene (Dmrt5)*. Preliminary RNA *in situ* hybridisation revealed restricted expression of *Dmrt5* in the ventral midbrain between E10 and E12 (Su Ling Zhao).

The identification of *Dmrt5* from a microarray screen, its localisation to the region from which dopaminergic neurons arise and the family's previous role in development provide evidence to support the hypothesis that Dmrt5 could be

involved in midbrain dopaminergic neuron development. To investigate this further, in the following results chapters I characterise the expression of *Dmrt5* in the developing chicken embryo in chapter 3, I describe the use of *in ovo* electroporation and an inducible EpiSC line to investigate the effect of *Dmrt5* over expression on neuronal differentiation in chapter 4. I discuss results from a microarray screen carried out to identify potential functions of Dmrt5 during neuronal development in Chapter 5.

Chapter 2. Materials and Methods

1.1 Materials

Unless otherwise stated, all analytical grade chemicals were obtained from either Sigma or BDH Laboratory Supplies (Merck Ltd). Stock solutions were prepared with reverse osmosis purified water and filtered and autoclaved as necessary. Agarose for electrophoresis was supplied by Invitrogen. Synthetic oligonucleotides were synthesised by SIGMA.

2.1.1 Solutions

Phosphate buffered saline (PBS):

137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4.7H_2O$, 1.4mM KH_2PO_4 (pH7.4)

PBST:

0.2% (V/V) TRITON X 100 (BDH) in PBS.

1X TAE:

0.04mM Tris-acetate, 0.001M Ethylenediaminetetraacetic acid (EDTA)

20X SSC:

3M NaCl, 0.3M Tri-Na Citrate

Lysis buffer:

10mM Tris (pH8.0), 50mM EDTA, 100mM NaCl, 0.5% sodium dodecyl sulphate (SDS), 0.5mg/ml Proteinase K (Roche) added just before use.

Luria-Bertani (LB) broth:

1% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract, 85mM NaCl.

LB Agar:

1.5% (w/v) agar (Difco) in LB broth.

ChIP lysis buffer:

0.1% Triton X-100, 0.4mM PMSF (SIGMA) in PBS.

MA buffer:

0.25M Sucrose, 3mM $MgCl_2$, 10mM Tris-acetate pH8.0.

ChIP Resuspension buffer:

4ml MA, 1ml 5x Complete protease inhibitor cocktail (Roche), 2mM $CaCl_2$

ChIP dilution buffer:

200mM NaCl, 1mM EDTA, 1%Triton X-100, 20mM Tris-acetate pH8.0.

ChIP elution buffer:

0.1M $NaHCO_3$, 1% SDS.

TBST

1x TBS and 0.1% Tween 20

MAB (pH7.5)

0.1M Maleic acid pH adjusted to 7.5 with NaOH, 0.15 M NaCl

MABT

MAB with 0.1% Tween-20

Blocking solution

MABT + 2% Boehringer Blocking reagent (Roche) + 20% FCS

NTMT

100mM sodium chloride (BDH), 100mM TrisHCl (Sigma) pH9.5, 50mM magnesium chloride (BDH), 0.1% Tween-20 (BDH).

4% Paraformaldehyde

17.5 grams paraformaldehyde (BDH), 500ml PBS heated to boiling, drops of 1M NaOH added until solution clears to adjust pH.

PBST

PBS with 0.1% Tween-20

Prehybridisation mix (Prehyb)

50% formamide (Fluka 47670)

1.3M SSC (use 20X SSC at pH5)

5mM EDTA

0.2% Tween-20

Hybridisation mix

Prehyb + 10% (w/v) CHAPS, 100ug/ml Heparin, 50ug/ml Yeast tRNA + 0.1mg/ml probe

2.2 Molecular biology methods

2.2.1 Transformation of competent cells

50µl aliquots of DH5α competent cells (Invitrogen) were thawed on ice and approximately 10ng plasmid DNA was added. Competent cells were gently stirred and placed on ice for 30 minutes. The tube was heated at 42^{o}C for 20 seconds and immediately placed on ice for 2 minutes. 1ml of LB broth was added and the tube was incubated in an automated shaker (Sorvall) for 1 hour at 37^{o}C. 20 - 200µl of the culture were plated on LB agar plates with appropriate antibiotic selection and incubated at 37^{o}C overnight.

2.2.2 Amplification of transformed bacterial colonies

Single colonies from the agar plates were picked using a 200µl plastic pipette tip (Star Lab) and transferred to a 15ml Falcon tube (Marathon) containing 3ml LB broth with appropriate selection. The culture was incubated in a shaker at 225-250 rpm at 37^{o}C overnight. The bacterial suspensions were used for mini prep (Qiagen).

2.2.3 Preparation of Plasmid DNA

Qiagen mini, midi and maxi-prep kits were used according to manufacturer's instructions to obtain desired quantities of plasmid DNA. For mini prep, 1.5ml of the culture was centrifuged at 13000 rpm for 2 minutes and the supernatant was

discarded. 150µl of solution 1 was added to the tube and vortexed. To lyse the cells 150µl solution 2 was added and the tubes were inverted 4-5 times to mix. To neutralise the lysate, 250µl of cold solution 3 was added and the tube was inverted 4-5 times to mix. The sample was then centrifuged at 13000 rpm for 10 minutes and the supernatant transferred to a clean tube. To precipitate the DNA, 1ml cold ethanol was added and the tube inverted 4-5 times to mix before incubating at -20^{o}C for 30 minutes to overnight. The samples were then centrifuged at 1300 rpm for 15 minutes to pellet the DNA, which was then dissolved in the appropriate volume of dH_2O to give the required concentration of DNA. Purified DNA was quantified using NanoDrop ND-1000 (Labtech).

2.2.4 Extraction and purification of RNA from chicken embryos and cultured cells

For cultured cells: Culture media was aspirated from cells, cells were washed twice with PBS. Cells were lysed using Tri-reagent (0.2ml per 2cm^2 well, Sigma). The lysate was transferred to a clean 1.5ml Eppendorff tube and vortexed. For chicken cells: embryos were collected and dissected as described in the preparation for microarray experiments section below. Dissected tissue from 6 embryos was put into one Eppendorff tube containing 0.5ml tri-reagent (Sigma). The tissue and tri-reagent were then vortexed to ensure all cells within the tissue lysed.

To extract RNA, 200µl chloroform was added and the vortexed for 15 seconds. The aqueous phase was collected and 500µl isopropanol added to this to precipitate the DNA. The tubes were incubated at room temperature for 30 minutes. Tubes were centrifuged at 12000 rpm for 10 minutes at 4^{o}C and the suspension removed. 1ml 75% ethanol was added to the pellet, and the tube was again centrifuged at 12000

rpm for 5 minutes. The supernatant was removed and pellet air-dried until clear. The pellet was then dissolved in required volume of RNase-free water. Purified RNA was quantified using NanoDrop ND-1000 (Labtech).

2.2.5 Reverse transcription

To generate cDNA from purified RNA, 1µg total RNA was mixed with 1µl Random Primers (3µg/µl, Invitrogen), 1µl dNTP mix (10mM, Invitrogen) and the appropriate volume of water to give a total volume of 13µl in a thin walled PCR tube (Fisher). The tubes were placed in a PCR machine and the following program was executed.

5 minutes at 65^{o}C

1 minute at 4^{o}C

Samples were removed from the PCR machine and 4µl 5x first strand buffer (Invitrogen), 1µl 0.1M DTT, 1µl RNAguard RNase inhibitor (27600U/ml, Amersham Bioscience) and 2µl Superscript III reverse transcriptase (200U/µl, Invitrogen) was added to each tube.

5 minutes 25^{o}C

60 minutes 55^{o}C

15 minutes 70^{o}C.

To remove the remaining RNA, 1µl RNase H (2U/µl, Invitrogen) was added and the tube was incubated at 37^{o}C for 20 minutes. The cDNA was diluted 1:10 in water before being used for quantitative polymerase chain reaction (Q-PCR).

2.2.6 Real-time PCR

For Real-time PCR 2µl of cDNA was mixed with 10µl MESA Green qPCR Mastermix Plus mix (Bio-Rad), 10mM each of the forward primer and reverse primer and 6µl water. Amplification was done for 40 cycles (94^{o}C 1min, 65^{o}C 1min, 72^{o}C 1min). Q-PCR was carried out using the Chromo 4 real-time PCR system (Bio-Rad).To verify the PCR product, melting curves were carried out in each reaction. Real-time PCR was performed in triplicates.

2.2.6.1 List of Q-PCR primers

Chick gene primers		
Genes	**Forward**	**Reverse**
GFP	ACGTAAACGGCCACAAGTTC	AAGTCGTGCTGCTTCATGTG
Beta actin	CACCATTGGCAATGAGAGG	GATTCATCGTACTCCTGCTTGC
18s RNA	GACACGGACAGGATTGACAG	GACAAATCGCTCCACCAAC
GAPDH	GAAAGGTCGCCTGGTGGATC	GGTGAGGACAAGCAGTGAGGAACG
Hes1	AAAGACAGCTCTCGGCACTC	CCTCGTTCATGCACTCATTG
Meis 2	CTCCTTCGACTCCTTCAACG	ACCGATGGCAGAAGTTATCG
Cald1	ACGGTTTAGCATCGACACTCC	ATGGAAGTGTTGAACGGGAG
GDPD5	TCCTCATCCTCACCTCATCC	AGGTTCATTTGCTGTCCCAC
arghef9	GGAGCGTTATCGCAAAGAAG	TGGTAAGTGGGTTCCTCCTG
Meis2	CTCCTTCGACTCCTTCAACG	ACCGATGGCAGAAGTTATCG
cxcl12	CAACTGCTCGCTTCAGATTG	TGGATCCATTTTAGCTTGGG
Cadm1	CAACATGCCGTACTGTCTGG	CCTGCTCGAGAATCGTATACATACAGC
Itgb5	TATGAAATGGCCTCAAACCC	TGTTGAACTTGTTGAAGGCG
Hes1	AAAGACAGCTCTCGGCACTC	CCTCGTTCATGCACTCATTG

Mouse gene primers	**Forward**	Reverse
Dmrt5	GCCTGCCTACGAAGTCTTTGGCTCGGTTT	CGTCTTGGGAAACAGATCAAACTTCTGCAATTT
HMBS	ACTGGTGGAGTCTGGAGTCTAGATGGC	GCCAGGCTGATGCCCAGGTT
Cyclophilin	GGCAAATGCTGGACCAAACAC	TTCCTGGACCCAAAACGCTC
GAPDH	GACCACAGTCCATGCCATCACT	TCCACCACCCTGTTGCTGTAG
Hes1	TCGGTGGGTCCTAACGCAGT	ACGGGTAGCAGTGGCCTGAG
Ngn2	GCTGTGGGAATTTCACCTGT	AAATTTCCACGCTTGCATTC
Mash1	CCCTGAAACTGGGTTGATGT	AAAGGCTGTCCGAGAACTG
Nato3	TCTGCGAGTTCCCACCTGGGG	TTTTGGGGCGGCCCAGCAAG
Cyclin D1	TTTCTCTGCCCGGCTTTGATCTC	CGCGCGGAGTCTGTAGCTCTC
PCNA	TTCGCTGCCGCTTCTGCATCG	TTGCGGTCGCAGCGGTATGTG
p21	GCAGCCGAGAGGTGTGAGCCG	ACGCGCTCCCAGACGAAGTTG
p27	ACAAGCGGAGCACCCCAAGC	GCAAGCTGCCCCTCTCCACC
chk1	GGCTTGGCAACGGTATTTCGGC	TCACTGGGCTGGTCCCACGG
chk2	TCGGTGACCCTCCCTGTGGC	GCCCCACACACAGACAGGCG
wee1	AGCCGCGGAACCCGAACTTG	GGCGGAGCACGGGAGAACAC
Socs3	CCAGCCTGCGCCTCAAGACC	GCGTGCTTCGGGGGTCACTC

2.3 Chromatin Immunoprecipitation

2.3.1 Preparation and fragmentation of chromatin

Approximately $1x10^7$ cultured cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature. 20ml Chromatin Immunoprecipitation (ChIP) lysis buffer was added and cells were removed from petri dish by scraping and transferred to a 50ml Falcon tube (BD Biosciences), cells were then centrifuged in Eppendorff 5810R centrifuge, at 2000 rpm for 5 minutes at 4^oC. The suspension was discarded and the pellet resuspended in 500µl ChIP Resuspension buffer. To fragment the chromatin, 0.5µl micrococcal nuclease (100U/ml, Roche) was added and tube was incubated at room temperature for 5 minutes. 25µl EDTA, 25µl 20% Triton X-100

and 25µl 20% SDS were added and the mixture sonicated with Sonics sonicator (Vibra Cell) for 2 minutes. The lysate was centrifuged at 12000 rpm for 15 minutes at 4 ^{o}C and DNA suspension transferred to a clean 1.5ml Eppendorff tube.

2.3.1.1 Testing chromatin fragmentation.

1µl RNAase A (10mg/ml, Invitrogen) was added to 50µl DNA suspension, this was then incubated for 10 minutes at room temperature. 450µl ChIP elution buffer and 20µl 5M NaCl were added and samples were incubated at 50^{o}C overnight. DNA was purified by addition of 500µl phenol/chloroform (1:1 v/v). The tubes were mixed and centrifuged at 12000 rpm for 5 minutes at room temperature. The aqueous phase was transferred to a clean eppendorff tube, 50µl 3M NaOAc and 500µl isopropanol were added and samples were incubated at -20 ^{o}C for 1 hour. The tube was centrifuged at 12000rpm for 15 minutes at 4 ^{o}C. The resulting pellet was washed with 1ml 75% ethanol and resuspended in 50µl TAE. To check DNA fragmentation, 5µl DNA was loaded onto a 1.5% agarose gel which was run at 90V for 1 hour. To assess the concentration of DNA in each sample NanoDrop was used.

2.3.2 Immunoprecipitation of fragmented chromatin

Chromatin samples were diluted so the concentration of all samples was the same using the required volume of ChIP resuspension buffer. 4.5ml ChIP dilution buffer and 500µl DNA suspension were added to a 15ml Falcon tube (BD Biosciences), samples were incubated on ice for 5 minutes. Samples were centrifuged at 12000 rpm for 15 minutes at 4 ^{o}C. 1ml supernatant (cleared chromatin); 40µl salmon sperm DNA/protein A agarose (SIGMA) and desired volume of antibodies were added to a clean 1.5ml eppendorff tube. Tubes were incubated overnight at 4 ^{o}C on a rotating wheel, and then centrifuged at 800rpm for 1 minute at room temperature. Following

careful removal of the supernatant agarose beads were washed 3 times with 1ml ChIP dilution buffer and two times in TAE. To elute DNA from the agarose beads, 500µl ChIP elution buffer was added to each sample. The samples were then incubated for 15 minutes at room temperature and then centrifuged at 800rpm for 1 minute. 20µl 5M NaCl was added to the samples which were then incubated overnight at 50 ^{o}C. The DNA was purified as set out in the previous section. The DNA was used for Real-time PCR.

2.3.2.1 List of antibodies used in ChIP experiments

Antibodies	**Amount for ChIP**
Histone H3 (ab1791, Abcam)	2µl
Trimethyl Lys 4 histone H3 (ab12209, Abcam)	10µl
Trimethyl Lys 27 histone H3 (07-449, Upstate)	5µl
Dmrt5 (custom made)	20µl
Non specific antibody (serum lab)	20ul

2.3.2.2 List of Primers used in Q-PCR analysis of ChIP experiments.

Primers	**Forward**	**Reverse**
p-2	AGGACATCAGGTTCTGTGCC	AGATTCCCCACAAGATTCCC
p0	ACATACAGAGTTCGAGCGGG	TTCTCTGGGCTTTGCTTAG
p2b	CGTGTCTCTTCCTCCCATTG	ATTCCGCTGTTATCAGCACC
p4	CCACCTCTCTCTTCTGACGG	AGGCGCAATCCAATATGAAC

2.4 Mammalian cell culture

Cells were grown in tissue culture plastics (Nunc) coated with 0.1% gelatine (SIGMA) and incubated at 37 ^{o}C with 5% CO^{2} in a humidified incubator (Sanyo). All solutions were sterility tested before use. To avoid bacterial and fungal

contamination, all tissue culture manipulations were carried out inside a laminar flow sterile hood (Nuaire) and surfaces and objects were sprayed with 70% ethanol before used.

2.4.1 EpiSC medium

100 mL Neurobasal Medium (Invitrogen, 21103)

100 mL DMEM/F12 (Invitrogen, 21331)

2 mL50x B27 (without vitamin A; Invitrogen, 12587-010)

1 mL100x N2 (Invitrogen, 17502-048)

200 µL 2-Mercaptoethanol (Invitrogen, 31350-010)

2 mL L-Glutamine(with penicillin/streptomycin;Invitrogen, 10378)

Add to EpiSC media before putting media on cells:

10 µg/mL FGF2 (Peprotech, 110-18b)

25 µg/mL Activin A (R&D, 338-AC-025)

2.4.2 Coating Plates

For passaging, 6-well plates were incubated with1ml FCS at 37°C for at least 1 hour. Before use, wells were rinsed twice with PBS. For EpiSC differentiation plates were coated with fibronectin (15 µg/mL) and incubated at 37°C for at least 1 hour. Fibronectin was aspirated off before plating cells.

2.4.3 Passaging

Cells were passaged once they reached ~80% confluency (or before they start to grow in 3D structures). Media was removed from the plate and cells were rinsed with PBS. For a well of a 6 well plate, 1 mL PBS was added per well and left for a few minutes until the cells started to round up. PBS was aspirated off, and 1 mL EpiSC media was added to the well. The bottom of the well was scratched with 5 mL pipette, back/forth in all directions to loosen as many cells as possible off of the bottom of the well. This method helps the cells remain in clumps, as they grow best this way in culture, rather than as single cells. Cell-media suspension was then triturated gently three times. Cells were then plated at three different densities. 6-well plate pre-treated with either FCS or fibronectin have 3 mL EpiSC medium added to each well. To each well containing 3mL EpiSC media,100, 200 or 300ul of the 1mL cell-media suspension was added.

2.4.4 Neural Differentiation

Once EpiSCs reach ~50-60% confluency, aspirate EpiSC media, rinse 1x (with N2B27 media) and add 3 mL N2B27 +PD (PD0325901, 1μM, Axon), to each well. Cultures are split at day 2 in the following manner: Rinse 1x with PBS, add PBS and let sit for a few minutes, aspirate, and add 1 mL N2B27 media. Scratch with a 5 mL pipette and transfer cells to a new 6 well plate pre-coated with fibronectin. Plate cells at 6 different densities depending on how dense you would like them. Typically, add 3 mL N2B27 to each well of a 6 well plate first, then add 200, 150, 100, 75, 50, 25 μL cells per well. After splitting, change media when needed (usually every other day). At day 5, 100ng/mL doxycycline was added to half the DA2-5 cultures to induced

exogenous Dmrt5 expression. Doxycycline was also added to half the tetracycline reverse transcriptional activator (rtTA) cultures as a control. Cells are then collected on day 6 or 7. Neurons appear between days 4-8.

2.5 Animal analysis

Fertilized chicken eggs were incubated at 37 ^{o}C for desired period of time to obtain the required stage of embryo.

2.5.1 *In ovo* electroporation

Hamburger-Hamilton (HH) st10 embryos were windowed and electrodes placed at either side of the developing head (CUY610P1.5-1, Sonidel Ltd.) [221]. A solution containing 7µg/µl DNA construct *pcaβ-IRES-GFP, or pcaβ-Hes1-IRES-GFP or pcaβ-Dmrt5-IRES-GFP*, 2% Polyvinyl alcohol, 0.05% Fast Green in water was injected into the developing midbrain. 5 x 12 volt square wave pulses of 50ms duration with an interval of 100ms were applied across the electrodes using an ECM830 Electro-S Square Porator (BTX lnc.). Following exposure to current, embryos were incubated at 37^{o}C for 24 hours; embryos were collected at the required stage. The procedure is depicted in Figure 2.1.

In ovo electroporation technique

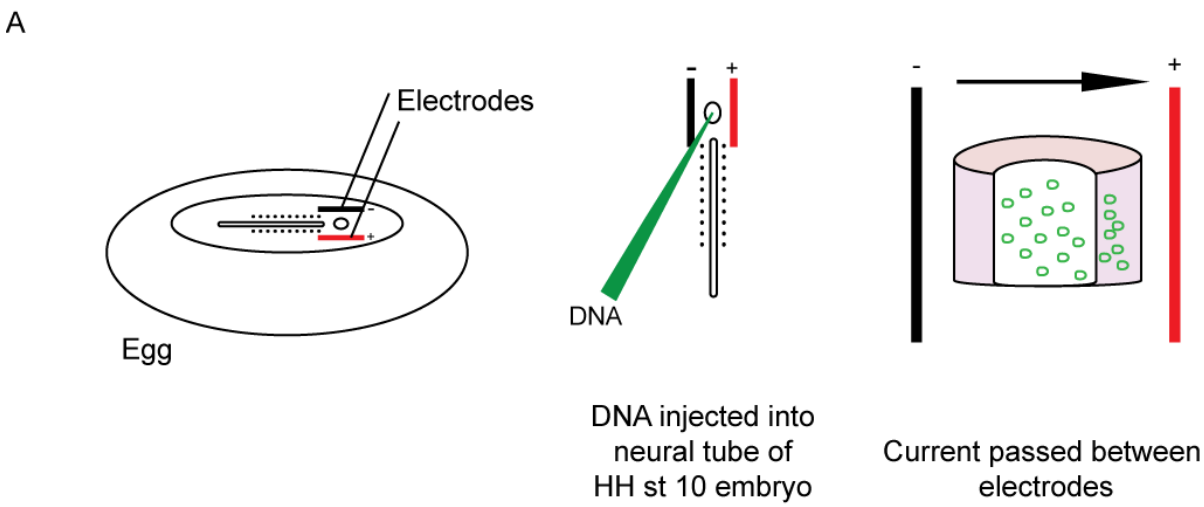




Figure 2.1. In ovo electroporation. **A**. Fertilised egg was incubated until it reached the required developmental stage. Egg is then windowed to visualise embryo. Electrodes are placed either side of developing anterior neural tube. DNA is injected into this region and a series of square wave electrical pulses are applied across the electrodes such that the DNA is taken up by cells adjacent to the positive electrode. **B**. HH st 16 embryos which were electroporated with *pcaβ-Dmrt5-IRES-GFP* 24 hours earlier. *In situ* for exogenous *Dmrt5*

(using mouse *Dmrt5* probe) has been carried out on the embryos, as can be seen the over expression is only on one side of the embryo.

2.5.2 Whole mount *in situ* hybridisation

Embryos were removed from eggs at desired stage, put into PBS or tyrodes solution and embryonic membranes removed. Cavities were opened; embryos were then fixed in 1-4% PFA for 1 hour-overnight depending on developmental stage. For this procedure unless otherwise stated, washes were carried out at room temperature and on rollers. Embryos were washed twice in PBST for 5 minutes, and then dehydrated through 25%, 50%, 75% MeOH/PBST and 100% MeOH. Solutions were changed when embryos sank to bottom of the tubes. Embryos were then bleached in 6% H_2O_2/MeOH for 15 minutes up to 1 hour depending on stage. Embryos were then placed in 100% methanol (MeOH) and stored at -20 ^{o}C overnight or until needed. To start *in situ*, embryos were rehydrated through methanol in the reverse order to PBST. Solutions were changed when embryos sank. Embryos were then washed in PBST for 5 minutes twice and placed in a solution of PBST containing between 10µg/ml – 1µg/ml Proteinase K (Roche) in PBST. Embryos were left in this solution without shaking for between 10-20 minutes depending on stage. Embryos were then placed in 4% PFA + 0.1% glutaradehyde (Roche) for 20 minutes. After this post fix embryos were washed in PBST twice for 5 minutes, and then rinsed in a 1:1 mixture of PBST/Prehybridisation buffer. The solution was changed and embryos were then incubated at 70 ^{o}C in prehybridsation buffer (prehyb) overnight. The next evening the embryos were placed in prewarmed hybridisation buffer containing approximately 1µg/ml probe. The following day embryos were rinsed in prehyb at 70 ^{o}C twice and then washed in Prehyb buffer at 70 ^{o}C for 20 minutes twice, during the second wash embryos were taken out of the 70 ^{o}C incubator and allowed to cool to

room temperature. Four 5 minute washes with MABT were carried out followed by two 30 minute washes with MABT. Embryos were then blocked with 2% BBR , 20% FCS in MABT for 1-2 hours at 4 ^{o}C, followed by incubation with Anti-DIG (1:2000, Roche) in 2% BBR 20% FCS in MABT overnight at 4 ^{o}C on a rocker. The next day embryos were rinsed in MABT for 5 minutes, four times followed by five 1 hour washes in MABT before being left overnight in MABT. The following day embryos were washed in NTMT twice for 10 minutes, they were then placed in colour reaction (3.5µl BCIP and 4.5µlNBT per 1ml NTMT), and at this point embryos were kept in the dark. Colour reaction was changed when solution turned purple or when embryos were sufficiently stained. Embryos were washed in NTMT for 10 minutes and then washed in MABT for 10 minutes or longer to reduce background. Embryos were then stored in PBST short term or 4% PFA for long term storage.

2.5.3 *In situ* hybridisation on sections

Frozen sections were allowed to equilibrate to room temperature. Labelled probe was diluted 1:1000 in hybridisation buffer and at least 300µl was placed on each slide. A coverslip was laid over this solution, and sections were incubated at 65 ^{o}C in a humidified chamber overnight. After hybridisation, coverslips were removed and sections rinsed twice in MABT for 5 minutes each. Sections were then washed in wash buffer at 65 ^{o}C for 30 minutes twice, followed by two 5 minute washes with MABT. Sections were then blocked with blocking solution for 1 hour. AP-Anti-Dig antibody was diluted at 1:500 in blocking solution and 500µl of this solution was added to each slide. Sections were incubated overnight at 4^{o}C in a humidified chamber. Sections were washed for three times in MABT for 10 minutes each. Sections were then washed in pre-development buffer for 2 minutes. Slides were

incubated at 37 ^{o}C in development buffer pH9.8, 5% PVA containing NBT and BCIP until sections acquired required intensity of blue colour. Reaction was stopped by washing slides in tap water. The sections were dried and mounted using a xylene based mounting solution. Sections were stored in the dark.

2.5.4 Cryostat sectioning

Embryos were dissected in tyrodes solution, and then fixed in 1-4% PFA in PBS for 1 hour. The embryos were then cryoprotected in 30% sucrose in PBS which was left at 4^{o}C until embryos sank. Embryos were then embedded in OCT (BDH) in embedding boxes or foil parcels over dry ice and stored at -80^{o}C until use. Cryosectioning was performed at -18 to -21^{o}C and cryostat and sections were cut at 100-5 µm. Sections were collected on polylysine slides and dried at room temperature for 30 minutes and then either used immediately for *in situ* or immunostaining or stored at -80^{o}C until use.

2.5.5 Immunohistochemistry

Frozen sections were allowed to equilibrate to room temperature. Sections were blocked with serum in PBST for 1 hour at room temperature and were then incubated with primary antibodies in blocking solution at 4^{o}C overnight in a humidified box. Sections were washed three times for 20 minutes with PBST at room temperature, then incubated with horseradish peroxidise (HRP) or fluorescence – conjugated secondary antibodies either at room temperature for 1 hour or overnight at 4^{o}C. Sections were then washed three times in PBS. For DAPI staining, sections were then incubated with DAPI (1:5000) for 3 minutes and washed three times in PBS. Sections were then mounted using Vector mount (Dabco) and analysed using

a Leica TCS SP5 confocal microscope equipped with 405 Diode, Argon, HeNe 594 and HeNe 633 lasers.

2.5.5.1 Primary antibodies used

Antibody	Host	Supplier	Concentration	Block
PH3	Rabbit	Abcam ab 32107	1 in 500	2% BSA 5% Serum 0.2% Triton X-100
DAPI dilactate		Invitrogen D3571	2mg/ml	PBS
Tuj1	Rabbit	Covance PRB-435P	1 in 1000	2% BSA 5% Serum 0.2% Triton X-100

2.5.5.2 Secondary antibodies used

Flurophore	Antigen	Host	Supplier	Concentration
Alexa 488	Mouse IgG	Donkey	Invitrogen	1 in 200
Alexa488	Rabbit IgG	Donkey	Invitrogen	1 in 200
Alexa 594	Mouse IgG	Donkey	Invitrogen	1 in 200
Alexa 594	Rabbit IgG	Donkey	Invitrogen	1 in 200

2.5.6 Tissue preparation for microarray analysis and real-time PCR

Embryos were collected and washed in tyrodes, and were transferred to clean tyrodes for dissection of the midbrain region. Dissected tissue was then treated with 2U/ml dispase (SIGMA) for 10-15 minutes. Mechanical dissection was used to remove the neural tube from the mesoderm. Ventral lateral midbrain (VLM) was dissected from wild type embryos without any treatment (VLM), embryos which had experienced electric current (VLMi), embryos exposed to current and *GFP* (VLMg) and embryos exposed to current and regulatory gene *Dmrt5* (VLMd). Tissue for real-time PCR of electroporated embryos was obtained by the same method.

2.5.7 Microarray

For each microarray 6 independent tissue samples of VLM, VLMi, VLMg or VLMd were collected and pooled. Total RNA was isolated from each pool using the Absolutely RNA Miniprep Kit (Stratagene). RNA was sent to UK Bioinformatics for processing and analysis. The procedure used was as follows: cDNA was biotin labelled. Samples were hybridized to GeneChip Chicken Genome Array (Affymetrix) according to manufacturer's protocol. For each condition three sets of pooled samples were collected and hybridised to three arrays Chicken Genome Arrays (Affymetrix) to obtain three biological replicates for each condition. Following Mas5, normalisation and filtering, genes whose expression differed significantly between control condition VLM and experimental conditions: VLMi, VLMg or VLMd were identified by one-way ANOVA and SNK post hoc test as outlined below.

2.5.8 Bioinformatics

Probe levels were calculated from raw data using Mas5 algorithm embedded into the GCOS suite (version 1.2; Affymetrix). Data were analysed using GeneSpring package (version GX11; Agilent Technologies, Wokingham, Berkshire, UK). The suitability of the expression data sets for inclusion in the analysis and the overall relationship between biological replicates was assessed using quantile plots and principle component analysis. Samples were first normalised to the 50^{th} percentile (median) across the entire expression data set. Genes were then filtered to remove any genes absent in all arrays. To identify genes showing differential expression between the three samples a one-way ANOVA and SNK post hoc test with no Multiple Testing Correction was carried out. A p-value of < 0.05 and a ≥ 1.5 fold

change in gene expression was used to determine genes showing differential expression between the samples.

2.5.9 Ingenuity network analysis

The differentially expressed genes were used to perform network analysis using Ingenuity Pathways Analysis v8.0-2602 (IPA) (http://www.ingenuity.com). The Ingenuity Knowledge Base is the largest knowledge base of its kind, with millions of findings curated from the full text literature. For analysis, the Affymetrix Chicken Genome Array gene list was used as a reference set. All data sources, all species, and all neuronal tissues and neuronal cell lines were used for the analysis. IPA uses a Fisher's exact test to determine which pathways are significantly enriched within the set of genes showing differential expression compared to the entire list of genes represented on the array.

Chapter 3. Expression of *Dmrt5* in Chicken embryos

3.1 Introduction

Dmrt5 is a member of the Doublesex and Mab-3 related transcription factor family. Dmrt family members contain a non-classical zinc finger like DNA binding motif known as the DM domain [222]. The DM domain is broadly conserved among metazoan family members [203]. This family of proteins has been shown to regulate sexual development in arthropods, nematodes and vertebrates [199, 200].

Although most investigations regarding the function of Dmrt genes have concentrated on sexual development [195, 198, 223], the presence of Dmrt gene expression in non-gonadal tissue [212, 219] presents the possibility of a role for Dmrt genes in development of other tissue types. Many members of the Dmrt gene family are expressed in the central nervous system (CNS) of mouse [195, 224], chick [224], Xenopus [225] and fish [211]. Dmrt genes have also been identified in the nasal placodes [211, 224], the presomitic mesoderm and newly formed somites [218, 226]. Whilst expression has been described in various tissues, the functional role of Dmrt genes outside of the gonads has remained largely un-investigated with some notable exceptions; Xenopus Dmrt4 has been shown to be an important regulator of neurogenesis in the olfactory system [225], whilst Dmrt2 is a critical regulator of vertebrate segmentation [217].

As described in Chapter 1, expression of *Dmrt5* in the ventral midbrain was detected in the Li Laboratory in a microarray experiment designed to find genes expressed in ventral midbrain dopaminergic neuron progenitors but not the more lateral tissue of E12 mice. In agreement with this finding, expression of *Dmrt5* has been observed in

the midbrain of Platyfish [211] and Zebrafish [227] and expression of *Dmrt5* has been observed in the brains of mice [195] and Fugu [223]. Table 3.1 provides full details of all published *Dmrt5* expression data.

Species	**Location**	**Detection Method**	**Citation**
Human	Testis	PCR	Ottolenghi et al., 2002 [198]
Mouse	Ovary, Testis, brain, kidney, heart, lung, stomach, adult testis	PCR	Kim et al., 2003 [195]
Mouse	Brain,	ISH	Kim et al., 2003 [195]
Platyfish	Nasal placode, lens, forebrain, midbrain, olfactory placode	ISH	Veith et al., 2006 [211]
Zebrafish	Ovary, Testis, midbrain, midbrain-hindbrain boundary	ISH	Guo et al., 2004 [212]
Fugu	Eye, brain, heart, spleen	Southern blot	Yamaguchi et al., 2006 [223]

Table 3.1. Published data describing *Dmrt5* expression during development. Table details species in which expression was detected, location of expression, method of detection and paper this work was published in. ISH = *In situ* Hybridisation.

To further characterise the expression pattern of *Dmrt5, in situ* hybridisation using a probe for Chicken *Dmrt5* was carried out in chicken embryos. We focused on describing the expression pattern of *Dmrt5* in the ventral midbrain as this region gives rise to midbrain dopaminergic neurons of the SNc and VTA [228].

3.2 Results

To determine if Dmrt5 is involved in midbrain dopaminergic neuron development and specifically in which process of midbrain dopaminergic neuron development the gene may have a function *in situ* hybridisation was used to locate *Dmrt5* RNA during the time at which midbrain dopaminergic neuron development occurs in chicken midbrain. To investigate the expression of chick *Dmrt5* by *in situ* hybridisation, a probe was made from an expressed sequence tag (EST) identified in a large scale EST project [229], which by Blast analysis showed a 99% identity and 98% coverage of the chicken predicted Dmrt5 mRNA (XR 026801.1). This EST was annotated as Chicken EST (ChEST) ChEST 44n20 and is a 767 nucleotide sequence.

Dmrt5 expression was detected in embryos from HH st 16 (equivalent to mouse E9.5), when specification to dopaminergic fate first occurs, up until HH st 34 (equivalent to an E14 mouse), when the majority of midbrain dopaminergic neurons have terminally differentiated. This time period covers the entire midbrain dopaminergic developmental process from specification, to neurogenesis and terminal differentiation. Analysis of whole mount chicken heads sagitally dissected show that in the chick, *Dmrt5* expression first occurs in the ventral midbrain at HH stage 16 at the ventral base of the Zona Limitans Intrathalamica (Figure 3.1). The expression of *Dmrt5* expands from this anterior position posteriorly from HH stage 16

to 34, with expression at its most posterior reaching the isthmus. Thus *Dmrt5* expression is seen in the region which gives rise to midbrain dopaminergic neurons during the time at which these neurons are born. Expression of *Dmrt5* is also seen in the dorsal telencephalon at HH st 16 up until at least HH st 34. (Figure3.1)

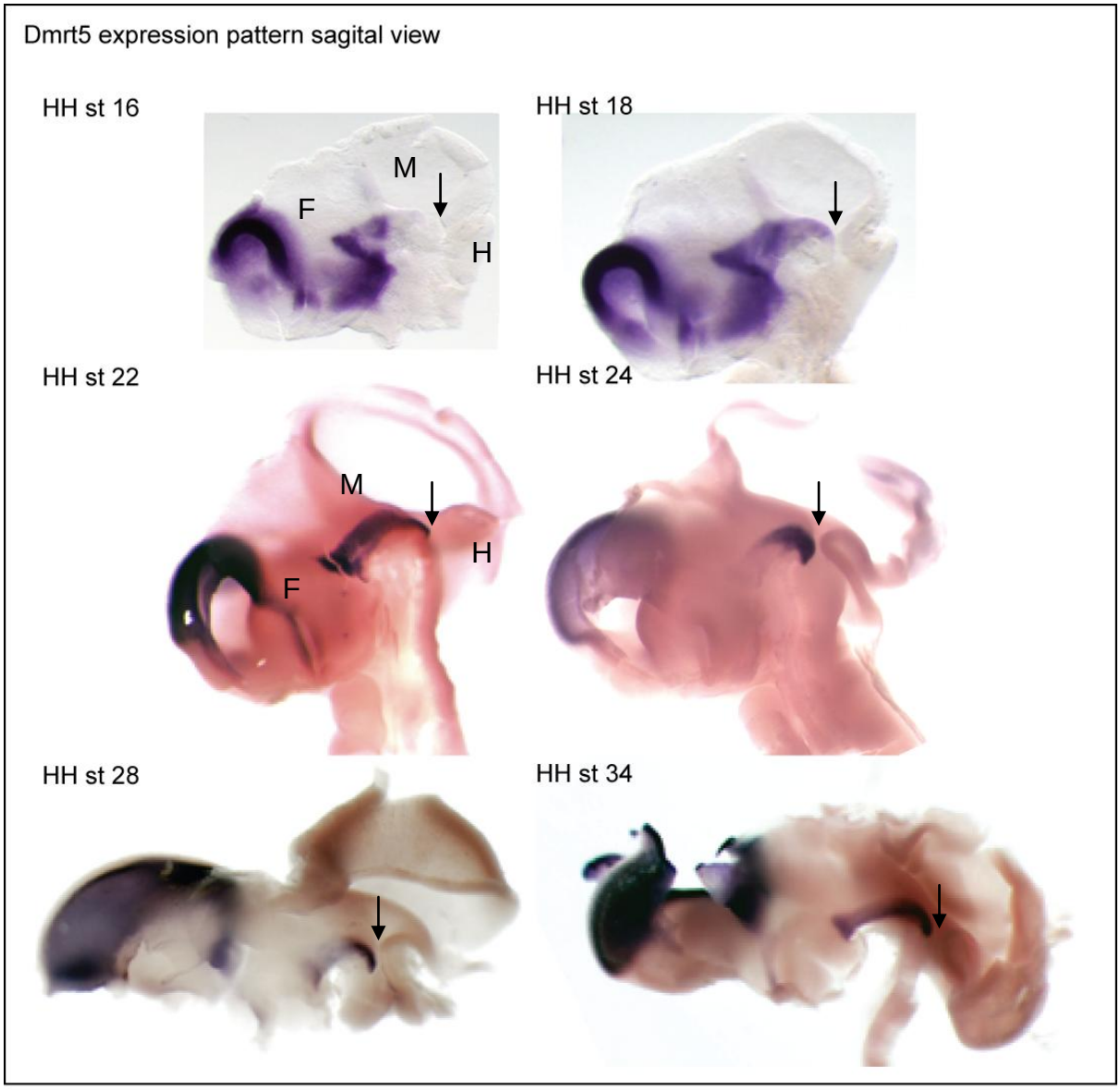


Figure 3.1. Expression pattern of Dmrt5 in developing chicken HH st 16 - 34. *In situ* hybridisation for Dmrt5 during development, embryos have been dissected sagitally orientated with forebrain on left and hindbrain on right. Arrow indicates midbrain-hindbrain boundary with midbrain to the left of the arrow and hindbrain to the right of the arrow. F shows forebrain region, M midbrain region and H hindbrain region.

Having identified expression of *Dmrt5* in the ventral midbrain during the development of midbrain dopaminergic neurons, I wanted to determine which layer of the developing ventral midbrain *Dmrt5* was expressed in. As described in Chapter 1,

during development the neural tube transforms from a single cell layer of neural epithelium into three layers. Each of the three layers contains cells in a different stage of midbrain dopaminergic development. By locating the cells that express *Dmrt5* we may be able to infer what possible role Dmrt5 could have during development of midbrain dopaminergic neurons and develop a suitable hypothesis to investigate the function of this gene further.

The three layers of the developing ventral midbrain are shown in Figure 3.2. They are the ventricular zone (VZ), which contains the proliferating progenitor pool [106, 107] and is the region where genes involved in specification are first expressed [115]; the intermediate zone (IZ), where cells begin to undergo neurogenesis and exit the cell cycle [114, 117]; and the marginal zone (MZ), where the fully differentiated neurons expressing TH and Pitx3 along with neuronal marker Tuj1 are located [141]. Coronal sections of HH stage 16 - 34 chicken embryos show *Dmrt5* RNA is present in the ventricular zone of the floor plate and a region of tissue immediately adjacent to the floor plate within the ventral midbrain throughout dopaminergic neuron development (Figure 3.2). While the in situ hybridisation images clearly show that Dmrt5 is expressed in the ventral midbrain it would be useful to use other probes such as corin and Lmx1A which mark the floor plate and Nkx6.1 which mark the more lateral fate, to obtain a more detailed understanding of the lateral limits of Dmrt5 expression.

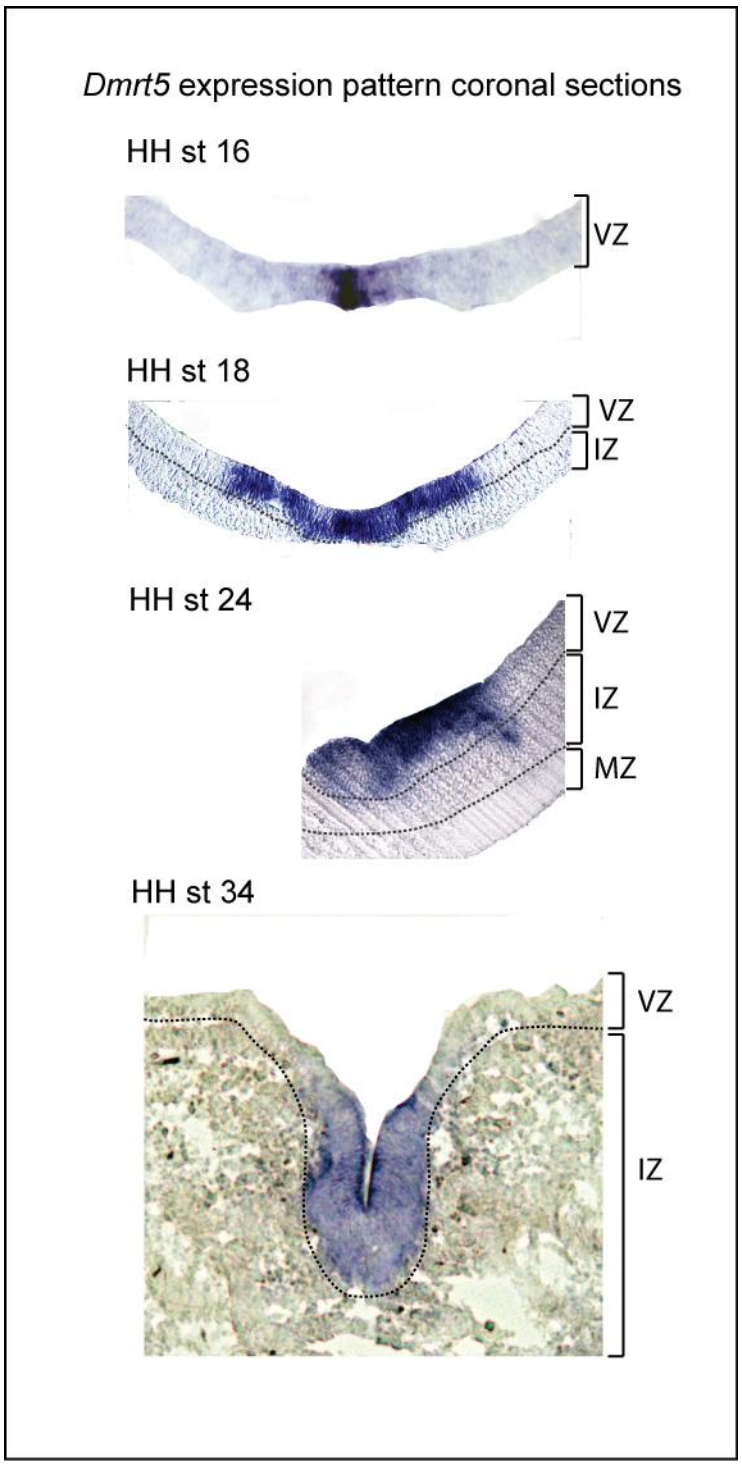


Figure 3.2. Coronal sections showing expression of *Dmrt5* in the ventral midbrain of chicken embryos HH st 16 - 34. *In situ* hybridisation of *Dmrt5* on coronal midbrain sections of chicken embryos, 3rd section is a sagital coronal. The three layers of the developing ventral midbrain are marked by a dotted line VZ = Ventricular Zone, IZ = Intermediate Zone, MZ = Marginal Zone. Zones are defined based on cell morphology. In situ experiments were carried out on at least 10 embryos per stage.

The RNA expression pattern of *Dmrt5* seen in the chicken embryo closely recapitulates the protein and RNA expression pattern of Dmrt5 seen during mouse midbrain development [230] (Emily Gale, unpublished data). This concurs with the high conservation of the Dmrt5 coding regions between mouse and chicken, which when compared by Blast show a 71% amino acid (AA) similarity (66% AA identity). Indeed Dmrt5 is one of the most conserved of the Dmrt gene family, with platyfish Dmrt5 showing 82% AA identity (88% similarity) with Zebrafish Dmrt5 and 57% AA identity (88% similarity) with human Dmrt5 [211]. A Dmrt5 homolog (AAA93409) is even found in C.elegans [227]. The conservation of Dmrt5 across 46 vertebrate species using UCSC genome browser was carried out (Figure3.3). This analysis was done by analysing base-wise conservation using a phylogenetic model, phyloP, from the PHAST package [231]. This program identifies sites predicted to be conserved which are assigned positive scores (shown in blue) while sites predicted to be fast evolving are assigned negative scores (shown in red), the absolute values of the score represent –log p-values and are relative to a null hypothesis of neutral evolution. The 46 vertebrate species used for analysis come from the reptile, amphibian, bird and fish clades as well as marsupial, monotreme (platypus) and placental mammals. As can be seen in Figure 3.3B the exons of Dmrt5 are highly conserved across the 46 vertebrate species.

In Figure 3.3C the individual alignment of chosen species compared to the human Dmrt5 gene can be seen with conserved regions shown in green. This conservation was calculated on UCSC genome browser using multiz alignment, a software program from the UCSC/Penn state bioinformatics comparative genomics alignment pipeline [232]. This analysis shows high conservation in the coding regions of human

Dmrt5 when compared to diverse range of the vertebrate family species from Monkey, through to Xenopus, Fugu and Zebrafish.

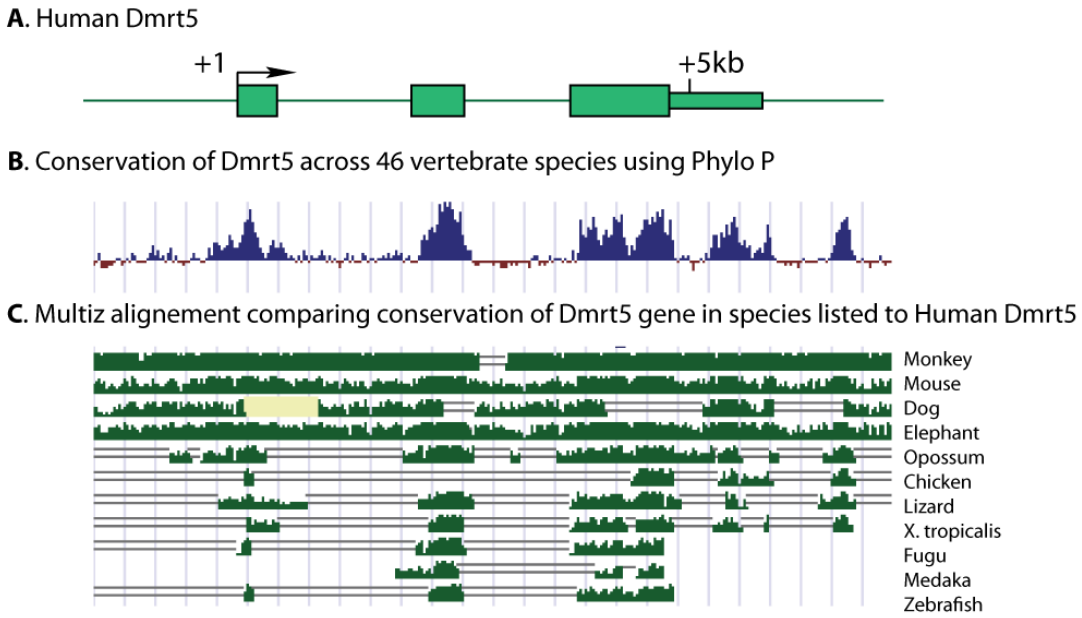


Figure 3.3. Conservation of Dmrt5 between species. Image created using UCSC genome browser showing **A.** Dmrt5 human gene **B.** The conservation of this gene between 46 vertebrate species, conservation is shown in blue and fast evolving regions shown in red. This was calculated using PhyloP which computes the conservation or acceleration p-values based on alignment and a model of neutral evolution. **C.** In green, the conservation between Human Dmrt5 gene and several vertebrate species, this was done using multiz alignment on the UCSC genome browser.

Outside of the midbrain, *Dmrt5* is expressed in the dorsal telencephalon (Figure 3.4A, C, D) which goes on to form the cerebral cortex, an area of the brain involved in memory, attention, perceptual awareness, thought, language and consciousness [233]. *Dmrt5* is also expressed in the developing eye which forms from the evagination of the diencephalon to give rise to optic vesicles [234]. Signalling

between this neural tissue and the overlying epithelial tissue leads to formation of the eye [235]. During eye development *Dmrt5* is expressed in the lens placode, a thickening of surface ectoderm which comes into contact with the optic vesicle. This lens placode has a role in formation of the optic cup [236] and gives rise to the lens and cornea [237-239]. Later in development, expression of *Dmrt5* is detected in the anterior region of the lens, and is expressed in the overlying epithelium which in combination with the migrating neural crest derived mesenchyme cells gives rise to the cornea [239]. The cells in the optic stalk also express *Dmrt5*, this expression is first detected at HH st16 and expression is still present in this region at HH st 34 (Figure 3.4A and B). The optic stalk is the proximal part of the optic vesicle, the ventral half of this invaginates and differentiates into glial cells and forms the tissue through which projecting retinal ganglion cell axons project, the dorsal half of the optic stalk gives rise to non neural tissue that encase the optic nerve [234] . Figure 3.4C shows expression of *Dmrt5* in the nasal placodes which later forms the olfactory epithelium of the nose [240] (Figure 3.4C). *Dmrt5* expression is also present in Rathke's pouch, which forms part of the pituitary gland a part of the endocrine system [241] (data not shown).

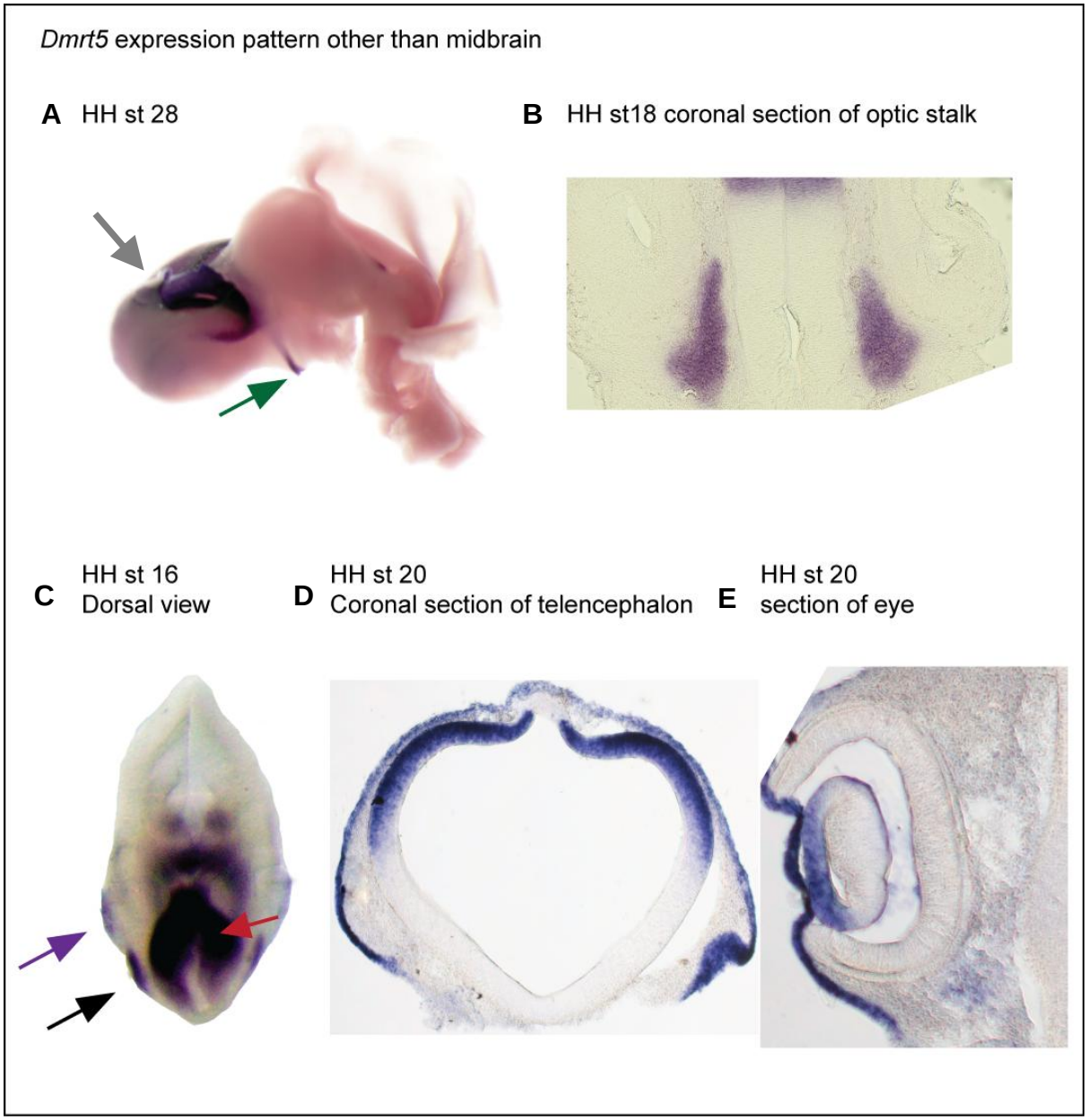


Figure 3.4. *In situ* hybridisation showing expression of *Dmrt5* outside of the ventral midbrain. A. HH st 28 embryo whole mount, *Dmrt5* expression in dorsal telencephalon (grey arrow) and optic stalk (green arrow) **B.** HH st 18 coronal section showing *Dmrt5* expression in optic stalk. **C.** HH st 16 embryo, dorsal view, showing *Dmrt5* expression in lens placode (purple arrow), nasal placode (black arrow) and dorsal telencephalon (red arrow). **D.** HH st 20 embryo, coronal section of telencephalon showing expression of *Dmrt5* in the dorsal telencephalon. **E.** HH st 20 coronal section of eye, *Dmrt5* is expressed in the mesodermal tissue overlying the optic cup and in the anterior lens.

3.1 Discussion

Dmrt5 is expressed in the ventricular zone of the ventral midbrain in a tightly spatially and temporally restricted manner from HH st 16 until at least HH st 34. As dopaminergic neurons start to undergo specification in this region at HH st 16, it is possible that Dmrt5 could have a role in midbrain dopaminergic neuron development. Furthermore, the temporal anterior to posterior expression pattern of *Dmrt5* from E9.5 onwards mirrors the way in which dopaminergic neurons are born as determined by emergence of *TH* expression during development [142].

The ventricular zone in the midbrain floor plate is the region in which the proliferating neural progenitors of dopaminergic neurons are found, and where expression of genes involved in specification to dopaminergic fate first occurs [106, 115]. Therefore it is possible that expression of *Dmrt5* in this region may have a role in maintenance of the progenitor pool or specification of these cells to dopaminergic fate. The conservation of *Dmrt5* expression pattern between mouse and chick, the identification of *Dmrt5* in the midbrain of Platyfish and Zebrafish, along with the strong sequence conservation between vertebrate species provide evidence to suggest that the function of this gene could be conserved.

Outside of the midbrain *Dmrt5* expression is detected in the nasal placodes, expression of *Dmrt4* [196] and *Dmrt3* [224] are also found in this region. We identified that *Dmrt5* is expressed in the dorsal telencephalon of mouse [unpublished data, Emily Gale] and chicken, similarly telencaphalic expression of Medaka and Xenopus *Dmrt4* [225], mouse and chicken *Dmrt3* [224] have also been observed. While, in the eye, expression of *Dmrt2* has been described [217]. Within the lab we have used *in situ* hybridisation and real-time PCR to detect the presence of other

Dmrt genes in the midbrain, so far we have not detected any other Dmrt family members in the ventral midbrain (Emily Gale).

In the future it will be interesting to identify if Dmrt5 has a conserved function in these different tissues. It has previously been proposed that Dmrt genes act as dimers, based on the Dmrt protein structure which contains a C-terminal domain dimerisation domain and *in vitro* studies which show dimerisation between family members [208]. As the expression pattern of the Dmrt genes is further investigated it will be interesting to see if co-localised genes such as those seen in the nasal placode (Dmrt5, 3 and 4) act as dimers [210]. However the focus of this thesis will be to investigate the function of Dmrt5 in the ventral midbrain.

Chapter 4. Investigating the role of Dmrt5 in midbrain development.

4.1 Introduction

In this chapter I will investigate if Dmrt5 has a role in neurogenesis of the ventral midbrain. This hypothesis was driven by evidence that two other DM domain family members, Dmrt4 in Xenopus and Mab-3 in C.elegans play a role in neurogenesis, via regulation of basic helix-loop-helix (bHLH) proneural genes, neurogenin and Ref-1 (a Hes homolog), respectively [215, 242]. Ngn2, Hes1 and Hes5 are involved in mouse ventral midbrain neurogenesis [114, 117, 243, 244]. Furthermore as described in Chapter 3, *Dmrt5* expression is temporally and spatially compatible with a role in ventral midbrain neurogenesis.

Neurogenesis within the ventral midbrain occurs from around E10 to E14 in mice, with the highest rate at E11.5 [126]. The neurogenic floor plate is characterised by proliferative cells within its ventricular zone and expression of proneural genes [114, 117, 127]. The generation of midbrain dopamine neurons requires the proneural gene Ngn2, which is expressed in the ventricular and intermediate zone [114, 117]. Mash1, which is also expressed at a lower level in the ventricular zone, is not required, but can partially compensate for loss of Ngn2 [117]. Expression of proneural genes and proliferation seem to be linked, as loss of Ngn2 prevents cells in the ventricular zone from undergoing cell cycle exit [114, 117]. Conditional loss of Liver X receptors α and β (Lxr α/β) also reduces ventral midbrain neurogenesis and cell cycle exit [245]. Several other genes which affect neurogenesis such as Otx2, Wnt2 and Nato3 also affect proliferation, further suggesting there is a link between these two processes [130, 139, 244].

Two signalling pathways, key to specification and patterning of the ventral midbrain, regulate expression of Ngn2 and cell proliferation, these are the Foxa2-Shh pathway and the Wnt1-Lmx1A pathway, these pathways are discussed in chapter 1 and illustrated in Figure1.4 [118, 121, 123-125].

Along with Shh-FoxA2 and Wnt1-Lmx1A mechanisms that appear to be involved in control of neurogenesis, Hes1 also seems to be involved in this process [243, 244]. Hes1, a bHLH protein, inhibits expression of proneural genes and is required for maintenance of neural progenitors [246-248]. Recently, Nato3 was found to be required for cell cycle progression and induction of proneural genes, Ngn2 and Mash1, in the ventral midbrain via repression of Hes1 [244]. How these findings correlate with previous understanding regarding the role of Otx2, Shh, Wnt, Lmx1A/B and Msx1 on neurogenesis is not known. Nato3 appears to be a direct target of FoxA2, as two FoxA2 binding sites are found within the promoter of Nato3 which are necessary and sufficient for promoter activity [249].

Further evidence for the role of Hes1 in the development of the ventral midbrain comes from experiments using conditional loss of FGF receptors 1 and 2 (FGFR1/2), which demonstrate that FGF signalling is needed for Hes1 expression in the ventral midbrain at E10.5 and E11.5. Loss of FGFR1/2 reduces Hes1 expression and results in premature neurogenesis and an increase in exit from the cell cycle [243].

In the following results section *in ovo* electroporation to exogenously express *Dmrt5* has been employed along with over expression of *Dmrt5* during dopaminergic neuronal differentiation of Epi Stem cells (EpiSCs) to investigate if Dmrt5 has a role in neurogenesis of the ventral midbrain.

4.2 Results

4.2.1 Dmrt5 affects expression of genes involved in neurogenesis

To explore the role of Dmrt5 in neurogenesis, HH st 10 chicken embryos were electroporated with a *pCaβ-Dmrt5-IRES-GFP* expression construct via *in ovo* electroporation. During *in ovo* electroporation, DNA is injected into the developing anterior neural tube. Electrodes are placed either side of the developing neural tube and a current is then passed across the tissue. This current disrupts the cell membrane and cells become transiently porous. The negatively charged DNA moves towards the positive electrode and is taken up via these pores by cells adjacent to the positive electrode. The contralateral side of the embryo serves as an internal control for exposure to the electroporation procedure. As a control for exogenous expression of DNA, stage matched embryos were electroporated with the same concentration of *pCaβ-IRES-GFP* construct.

Following *in ovo* electroporation at HH st 10 embryos were left to develop. No change in morphology was observed in embryos 24 hours post electroporation of *Dmrt5*. However, 48 hours after electroporation, the side of the midbrain exogenously expressing *Dmrt5* was much smaller and thinner than the contralateral control side. This effect was not seen when embryos were electroporated with *pCaβ-IRES-GFP.*

To characterise these changes in morphology, midbrains from embryos electroporated with *pCaβ-Dmrt5-IRES-GFP and pCaβ-IRES-GFP* at HH st 10 were collected at HH st 26, embedded in OCT and cryostat sectioned at 20µm. Dmrt genes have been found to be involved in regulation of neurogenesis in C.elegans, Xenopus and Drosophila [225, 242, 250]. To see if *Dmrt5* exogenous expression

effects neurogenesis, immunofluorescence was performed on midbrain coronal sections using an antibody specific for early pan-neuronal marker neuron-specific class III β-tubulin (Tuj1). *Dmrt5* over expression resulted in a reduction in the number cells positive for the neuronal marker Tuj1 relative to the control on the contralateral side (Figure4.1A). Over expression of *GFP* has no effect on the number of Tuj1 positive cells when compared to its contralateral side (Figure4.1B).

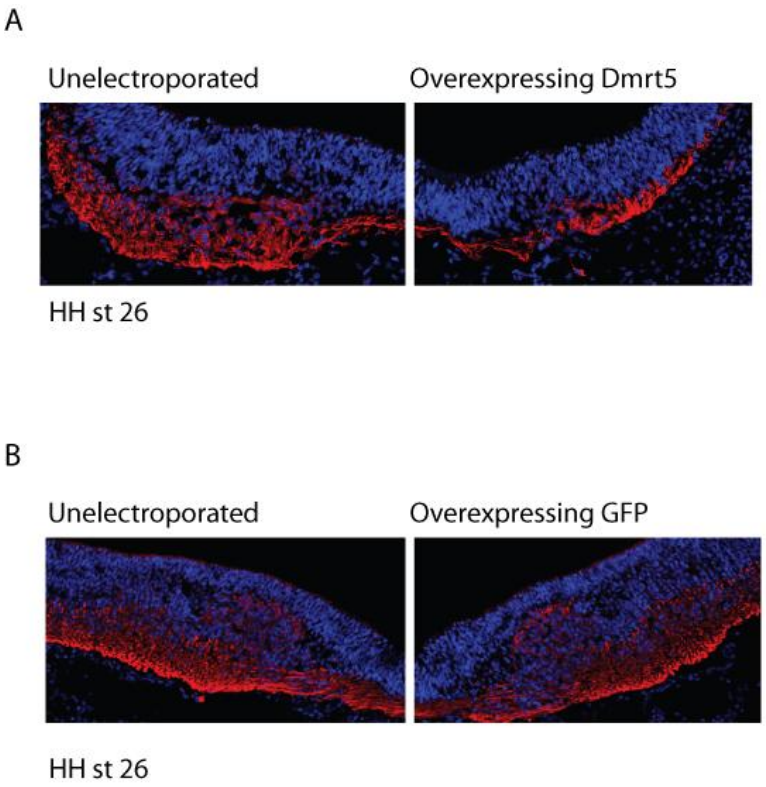


Figure 4.1. Ventral midbrain coronal sections of HH st 26 embryos electroporated at HH st 10 with **A.** *pCaβ-Dmrt5-IRES-GFP* **B.** *pCaβ-IRES-GFP*, stained with Tuj1 (red), a pan neuronal marker, and DAPI (blue).

To further characterise the apparent defect in neurogenesis and to confirm this is conserved between species, a mouse EpiSC cell line containing a *Dmrt5* transgene under the control of a doxycycline inducible promoter (this cell line will be described as the DA2-5 cell line hereafter), was differentiated towards neuronal fate using the monolayer differentiation protocol developed in our laboratory [187] and described in Chapters 1 and 2.

The expression of *Dmrt5* during this EpiSC differentiation protocol in the uninduced DA2-5 cell line can be seen in Figure 4.2. During dopaminergic differentiation exogenous expression of *Dmrt5* was induced on day 5 by addition of doxycycline. At this time point the cells endogenously express moderate levels of *Dmrt5* and therefore should contain any co-factors required for the function of this gene.

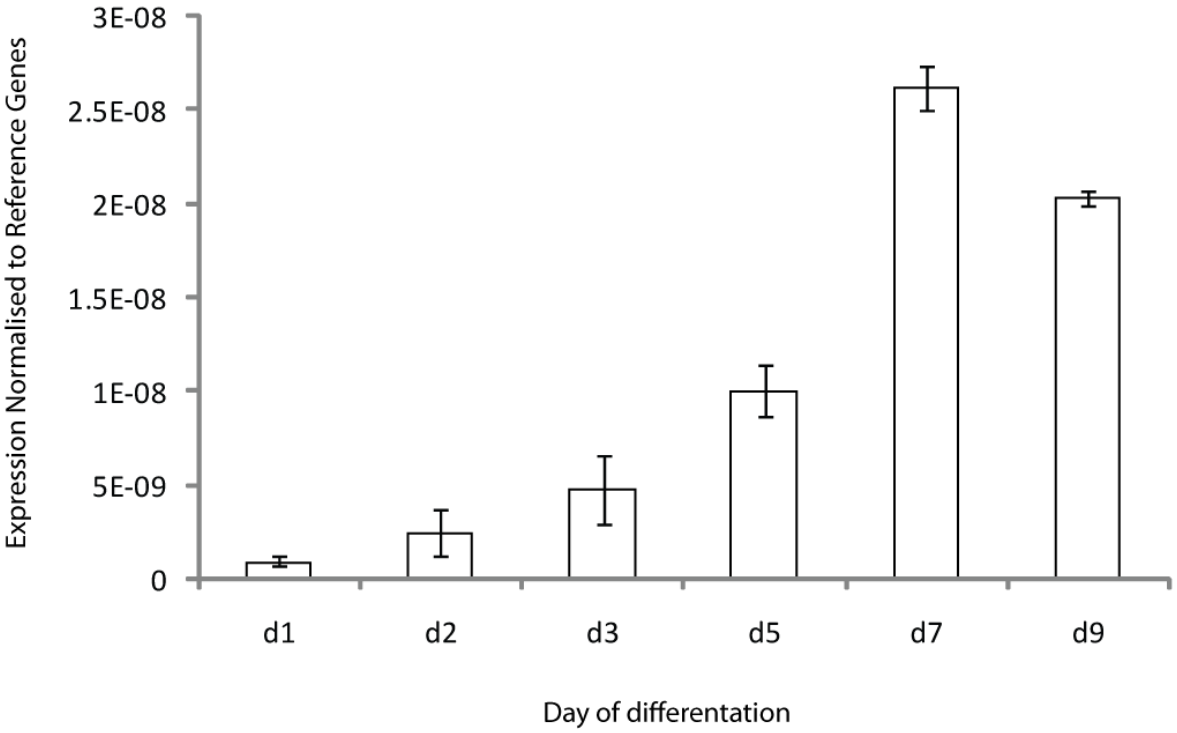


Figure 4.2. Expression of *Dmrt5* during monolayer differentiation of EpiSC*s* quantified by real-time PCR. Bars are the average of 3 biological replicates repeated in triplicate, error bars show standard deviation of biological replicates. Expression is normalised to reference genes, HMBS, Cyclophilin and beta actin. Data provided by Ines Jaeger**.**

EpiSCs differentiated to dopaminergic fate and induced to exogenously express *Dmrt5* on day 5 were collected 48 hours later, on day 7 of differentiation. Changes in gene expression between uninduced DA2-5 sister culture and induced DA2-5 samples were analysed by real-time PCR. A fourfold increase in *Dmrt5* expression in the induced DA2-5 cell line relative to the uninduced DA2-5 cell line resulted in a reduction in the expression of proneural markers *Ngn2*, *Mash1,* and the Hes1 repressor, *Nato 3.* Basic Helix-Loop-Helix (bHLH) transcription factor *Hes1,* which represses expression of proneural genes was up-regulated relative to the uninduced control. These findings are consistent with a reduction in neurogenesis [243, 244, 251]. (Figure 4.3)

No change in the expression of *Ngn2, Mash1, Nato3* or *Hes1* were detected upon addition of doxycycline to a non-inducible, control (rtTA) cell line relative to the rtTA cell line not exposed to doxycycline (Figure 4.3). Therefore the changes in gene expression observed upon addition of *Dmrt5* over expression were not a result of the addition of doxycycline.

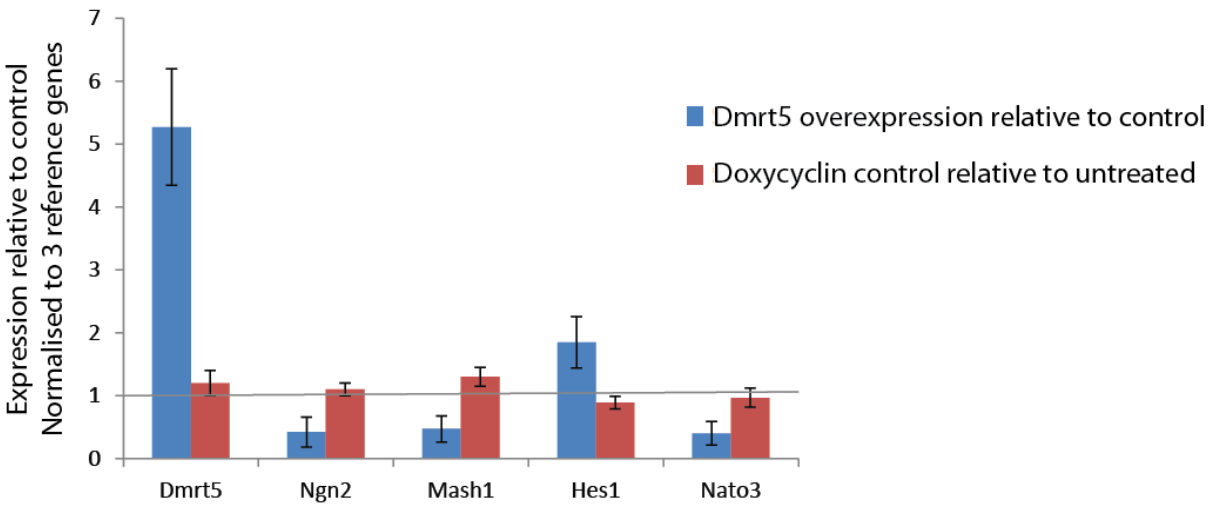


Figure 4.3. Expression of *Dmrt5* and neuronal genes in doxycycline induced DA 2-5 cell line relative to DA 2-5 uninduced (blue). Non-inducible control rtTA cell line exposed to doxycycline relative to rtTA cell line not exposed to doxycycline (red). Expression is normalised to reference genes, HMBS, Cyclophilin and beta actin. Data provided by Ines Jaeger**.**

The role of Hes1 in inhibition of neurogenesis has been well described. It is known to reduce levels of both Ngn2 and Mash1. Therefore the effects of *Dmrt5* over expression on neurogenesis could be mediated by an up regulation of *Hes1*. To determine if Dmrt5 could be involved in regulation of Hes1 *in vivo*, the expression pattern of both genes was mapped by *in situ* hybridisation. *Dmrt5* and *Hes1* are both expressed in the ventral midbrain at HH st 16 (equivalent to E9.5 in mouse) (Figure 4.4A). To investigate if *Dmrt5* could up regulate *Hes1 in vivo,* and to confirm that this regulation was conserved in both mouse and chicken, HH st 10 embryos were electroporated with *pCAβ-Dmrt5-IRES-GFP*, incubated for 24 hours, and stained for *Hes1* by *in situ* hybridisation. Exogenous expression of *Dmrt5,* as visualised by expression of GFP from the electroporated construct, led to ectopic expression of *Hes1* (Figure 4.4B).

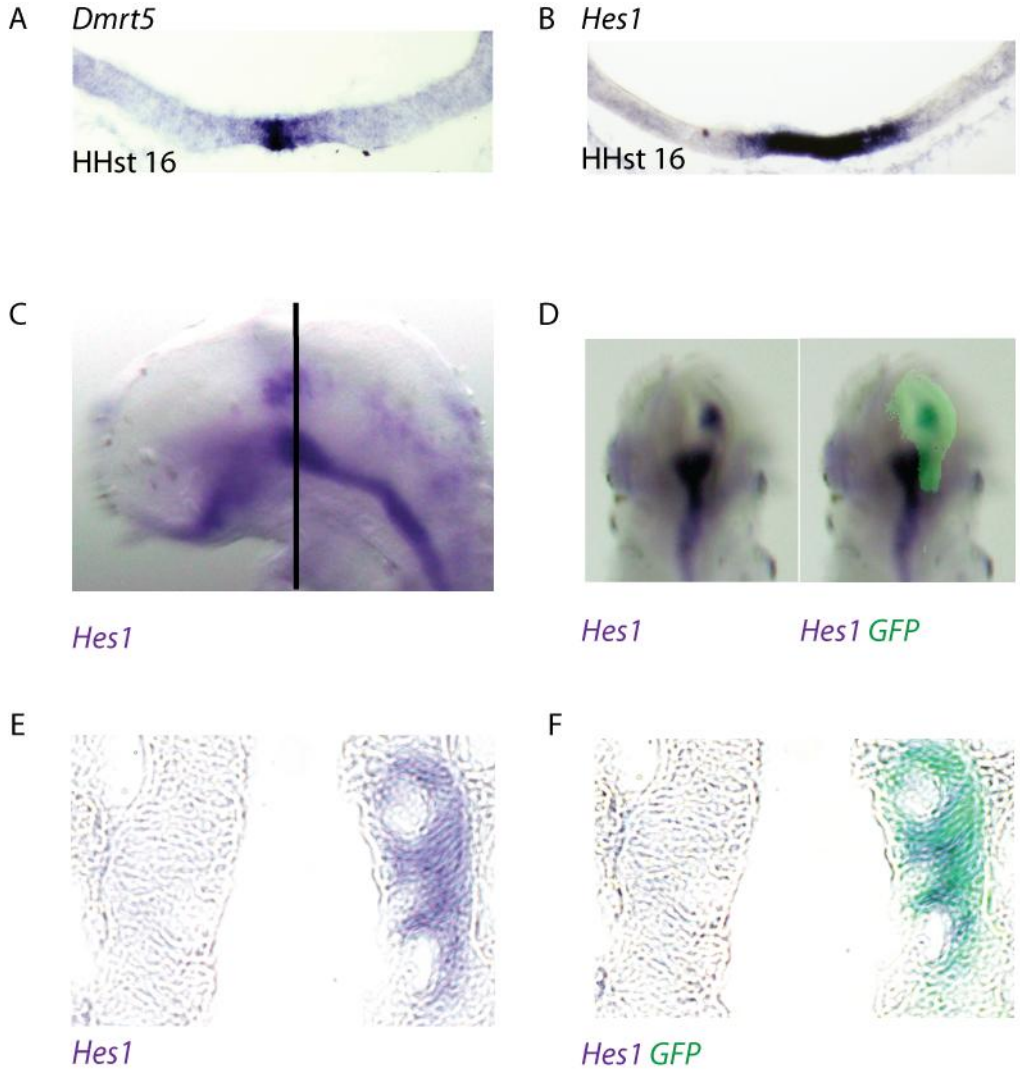


Figure 4.4. Dmrt5 affects expression of *Hes1 in vivo*. A. *In situ* hybridisation of *Dmrt5* in coronal section of ventral midbrain, HH st 16 embryos. **B.** *In situ* hybridisation of *Hes1* in coronal section of ventral midbrain, HH st 16 embryos. **C.** A whole lateral view of HH st 16 embryo electroporated with *pcaβ-Dmrt5-IRES-GFP.* Black line indicates angle of section used to obtain E and F **D.** Whole mount dorsal view of HH st 16 embryonic head electroporated with *Dmrt5* at HH st 10. **E and F.** Coronal section of HH st 16 midbrain of embryos electroporated with *pcaβ-Dmrt5-IRES-GFP* and stained for *Hes1*. *Hes1* expression is shown in purple, *Dmrt5* over expression is shown in green by *in situ* for *GFP*

4.2.2 Dmrt5 affects cell proliferation

Higher levels of Hes1 affect proliferation, maintain cells in a G1 retarded state and prevent neurogenesis [246, 251-253]. As *Dmrt5* exogenous expression induced

ectopic *Hes1* expression and caused a reduction in neurogenic gene expression, I investigated if *Dmrt5* also had an effect on cell cycle.

HH st 10 embryos were electroporated with *pCAβ-Dmrt5-IRES-GFP*, *pCAβ-Hes1-IRES-GFP* or *pCAβ-IRES-GFP* constructs, and collected 24 hours later at HH st 16. The midbrains of these embryos were then embedded in OCT and sectioned by cryostat at 12µm per section. To determine if *Dmrt5* exogenous expression alters the cell cycle and to confirm the previously published effects of Hes1 over expression on cell cycle, immunofluorescence was performed on midbrain coronal sections using an antibody specific for Phospho-Histone H3 (Anti-PH3). Phosphorylation of histone H3 requires active cdk1 and is an initial event accompanying chromosome condensation [254, 255]. As such Anti-PH3 specifically labels chromosomes from prophase through anaphase, and therefore it is a marker for entry into mitosis [254]. The proportion of PH3+ cells in the *Dmrt5*, *Hes1* or *GFP* electroporated-cell population, as marked by GFP expression, were quantified using sectioned midbrains of 6 embryos. As shown in Figure 4.5, both *Dmrt5* and *Hes1* over expression resulted in a decrease of PH3+ cells when compared to embryos electroporated with *GFP,* indicating a reduced number of cells going through mitosis under these conditions.

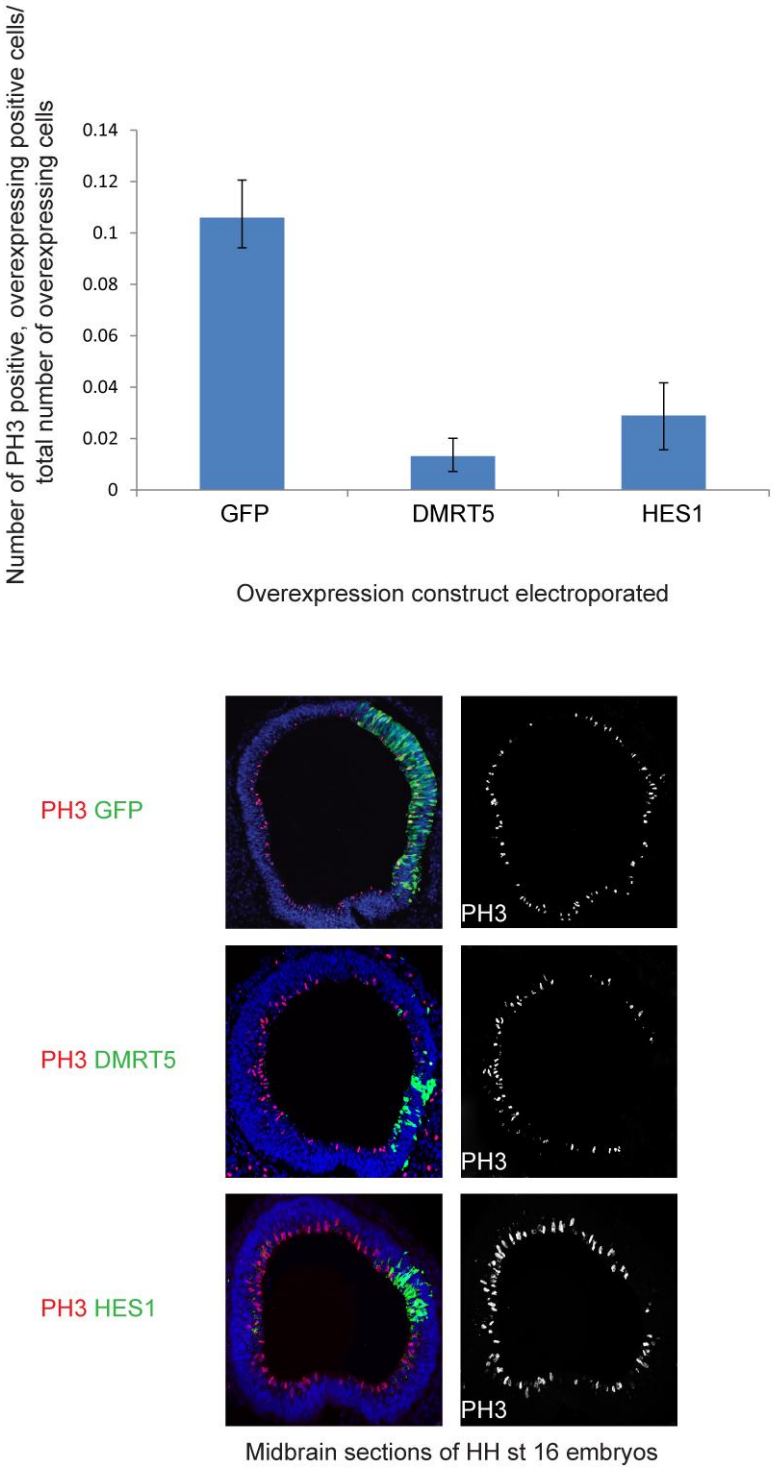


Figure 4.5. Effect of *Hes1* and *Dmrt5* exogenous expression on mitosis. A. Percentage of PH3+ electroporated cells, in embryos electroporated with *pCaβ-IRES-GFP*, *pCaβ-Dmrt5-IRES-GFP* or *pCaβ-Hes1-IRES-GFP*. Counting is based on midbrain sections from 6 embryos per condition. The average number of GFP expressing cells counted per embryo was 312. **B.** HH st 16 coronal sections of midbrain with PH3 staining in red and exogenous expression of the electroporated construct in green. Error bars show standard deviation.

To better understand the effect of Dmrt5 on cell cycle regulation, embryos were electroporated at HH st 10 with *pCaβ-IRES-GFP* or *pCaβ-Dmrt5-IRES-GFP*. At HH st 16 embryos were collected and electroporated cells, expressing GFP, were isolated and stained for propidium iodide (PI). (Figure 4.6)

PI is a fluorescent dye that intercalates stochiometrically with RNA and DNA within the cells. In the presence of RNAase, the stained cells incorporate an amount of dye proportional to their DNA content. The fluorescence is detected and is considered a measurement of cellular DNA content. As cells in different stages of the cell cycle contain differing amounts of DNA this can be used to identify what stage of the cell cycle the cells are in. As G2 and M phase both have identical DNA content these cannot be discriminated between based on DNA content. Therefore this analysis can only group cells into G1, S or G2-M stages. Cell doublets will report twice as much DNA as a single cell. Therefore these and other aggregates need to be removed from the analysis. This is done by setting up a series of gates that select only the cells which pass certain criteria, such that only the single cells are analysed.

The selection of single cells is based on several parameters: cell volume as measured by forward side scatter and the complexity of the cell (shape of nucleus, type of cytoplasmic granules, membrane roughness) by side scatter. The analysis based on side scatter area and forward scatter area allows selection of the cell population and removal of debris. Analysis of forward scatter area and width depicts doublets and aggregates so that one can select the singlet population. Doublets, aggregates and cell debris can also be filtered out of this population by comparing

the width and area of the fluorescent signal. Only cells which are selected as singlets by all these parameters were used for the analysis.

The PI staining of *GFP* positive or *Dmrt5* positive single cell populations were recorded by flow cytometry. While cells over expressing *GFP* showed a cell profile similar to cells that had not been electroporated, over expression of *Dmrt5* caused an increase in the number of cells to be held in G1 (Figure 4.6).

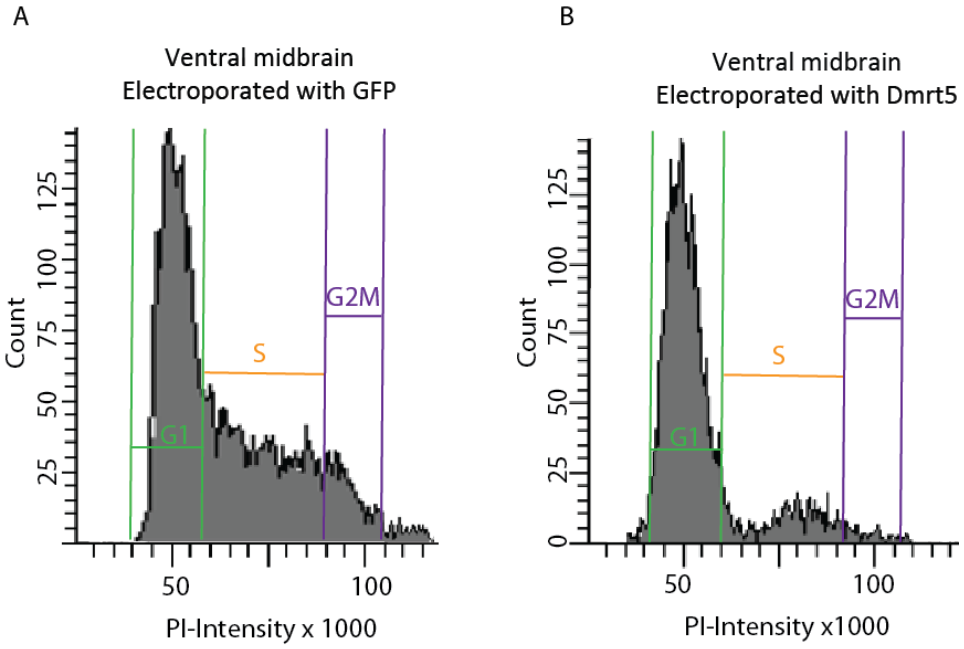


Figure 4.6. Effect of *Dmrt5* over expression on cell cycle. **A.** PI staining profile of ventral midbrain cells electroporated with *pCaβ-IRES-GFP* **B.** PI staining profile of ventral midbrain cells electroporated with *pCaβ-Dmrt5-IRES-GFP.*

Obtaining large numbers of cells from electroporated embryos for PI analysis is difficult. Therefore, the cell cycle profile of EpiSCs differentiated towards neuronal fate was measured. As larger numbers of cells can be obtained the defects in cell

cycle could be quantitated. *Dmrt5* over expression was induced in DA2-5 EpiSCs by addition of doxycycline on day 5; cells were then harvested at day 7 and stained for PI. The cell cycle profile of the doxycycline induced and uninduced DA2-5 cultures were compared using Flow Jo (Figure 4.7A). The difference in cell cycle profiles was calculated using the mathematical Dean Jett-fox model (Figure 4.7B). This model fits Gaussian curves to the G1 and G2 phases and a polynomial function to the S phase. The experiment was conducted using three biological replicates, error bars show standard deviation between biological replicates (Figure 4.7C). I found that a 5-fold up regulation of *Dmrt5* transgene expression caused 10% increase in the proportion of cells in G1 (Figure 4.7C, D). This G1 retardation phenotype is also seen when *Hes1* is over expressed in the mouse E13 telencephalon [251].

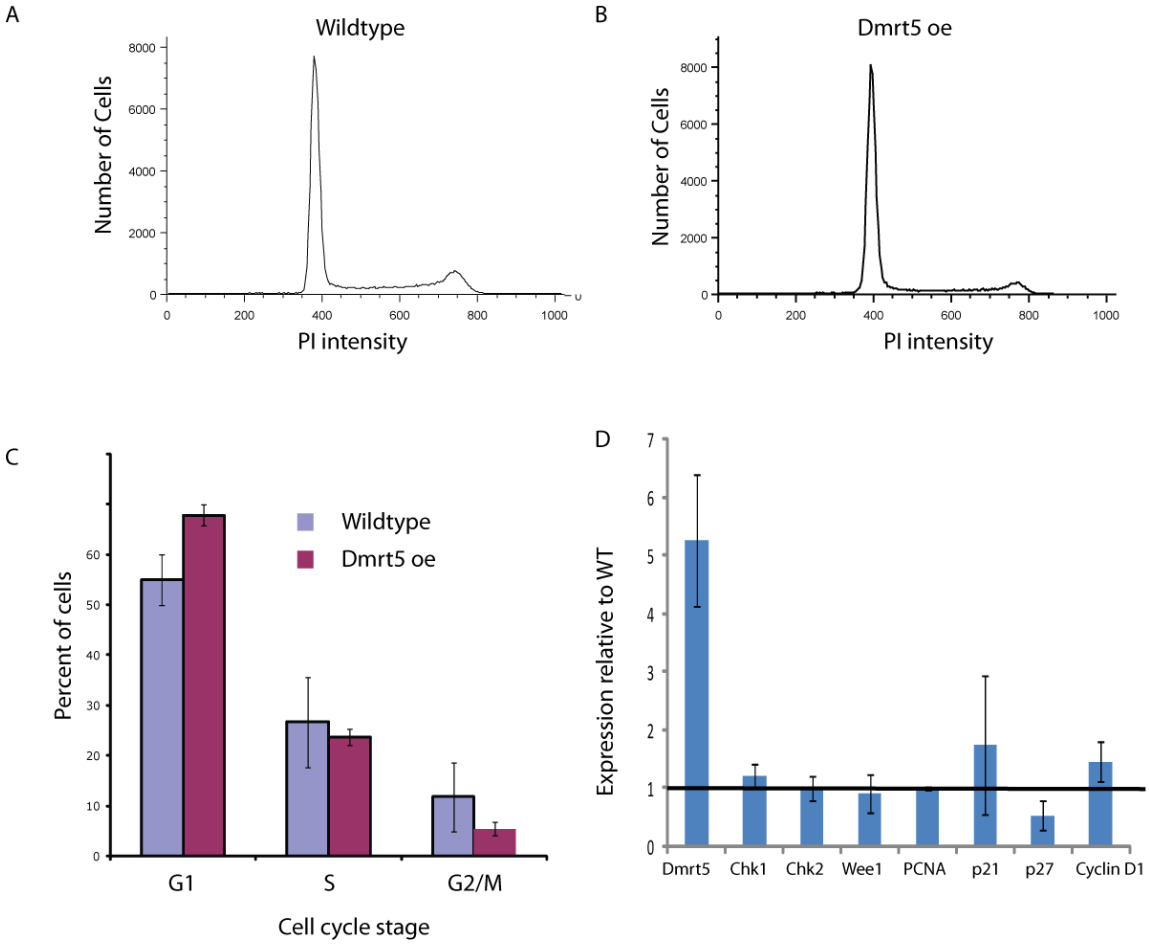


Figure 4.7. Effect of *Dmrt5* over expression in EpiSCs on cell cycle. A. PI staining of uninduced/wild type DA2-5 EpiSCs **B.** PI staining of DA2-5 induced cell line, over expressing *Dmrt5* **C.** Quantification of cell cycle analysis using mathematical model Dean Jett-Fox. Bars are the average of three biological replicates, error bars show standard deviation. **D.** Real-time PCR analysis of genes involved in cell cycle regulation. Expression in *Dmrt5* over expressing DA2-5 EpiSCs is normalised to the level of these genes in wild type uninduced DA2-5 cells. Bar represents average of 3 biological replicates normalised to three control cell lines, error bars depict standard deviation.

As the cells appear to be G1 retarded upon *Dmrt5* exogenous expression, the expression of genes involved in cell cycle regulation were analysed in the induced and uninduced DA2-5 cell line by real-time PCR. Induction was carried out on day 5 and cells were collected day 7. There was little change in S phase marker

Proliferating Cell Nuclear Antigen (PCNA) [256], or in G2/M checkpoint genes, Wee1 [257, 258], Chk1 [259] or Chk2 [260] expression upon *Dmrt5* over expression.

Cyclin D1 is a G1 phase marker, up regulation of this protein has previously been described in Hes1 over expressing G1 retarded cells [246, 261]. In line with this literature *Dmrt5* transgene expression leads to an increase in *Cyclin D1*. However, this gene has also been described to be down regulated upon over expression of *Hes1* [251]. p21 is a potent cyclin-dependent kinase inhibitor that binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1. Previously *p21* has been reported as up regulated in response to *Hes1* over expression [261]. However, I did not observe a consistent change in *p21* expression in response to *Dmrt5* over expression. There were large error bars for this gene as different samples had different levels of this gene but this was not a result of the Dmrt5 over expression. *p27* is another cyclin-dependent kinase inhibitor that can be directly inhibited by Hes1 [262]. While in cortical progenitors, expression of p27 has been shown to stabilise expression of Ngn2 and promote neurogenesis [263]. I detected a reduction in *p27* upon *Dmrt5* over expression. The same effect is observed upon *Hes1* over expression [27] (Figure 4.5D). These changes in *p27* and Cyclin D1 were seen when only 10 % of the cells were G1 retarded and with only a 5 fold upregulation of Dmrt5. It would be interesting to investigate the change of gene expression occurring in vivo where the effect of G1 retardation is far greater.

4.2.3 Does Dmrt5 directly control transcription of Hes1?

In C.elegans, Dmrt5 homolog, Mab-3, binds to the promoter region of Hes homolog, *Ref-1* [207]. I therefore postulated that Dmrt5 may induce expression of *Hes1* via binding to *Hes1* regulatory elements upstream of its transcriptional start site. To investigate this hypothesis, I searched for Dmrt5 consensus sequence binding sites in the mouse *Hes1* coding sequence and 10kb upstream of this. Two Dmrt5 consensus binding sequences were found just upstream of the *Hes1* promoter region, p0 and p2b (Figure 4.8A). ChIP experiments were used to identify if Dmrt5 could bind to these sequences.

DA2-5 EpiSCs were differentiated towards neuronal fate. For one experimental condition *Dmrt5* over expression was induced on day 5, by addition of doxycycline. As controls for the addition of doxycycline, the uninduced DA2-5 cultures that express endogenous levels of *Dmrt5* were used. As a further control, EpiSC*s* homozygous for a predicted Dmrt5 null allele were differentiated using the same protocol.

Cultures from the three cell lines were collected on day 6 and the proteins bound to the DNA were cross linked such that these interactions were fixed. The protein-DNA complexes were then extracted from the cells and this chromatin was sonicated to obtain 200-800 bp DNA-protein fragments. This fragmented chromatin is known as the starting material or input. The input is used to calculate how much DNA is initially present in each sample and therefore what proportion of the original input is pulled down during ChIP. This measurement is called the % input. For the ChIP experiment 500µl input chromatin was incubated with an antibody specific for Dmrt5. This precipitates any DNA to which Dmrt5 protein is cross linked. The protein-DNA cross

links are reversed and the DNA fragments are analysed. The amount of DNA isolated by this pull down is compared to the starting amount of this DNA in the input. To control for non-specific binding, input chromatin from each condition was incubated with a non-specific antibody. To control for DNA binding to the beads used during the precipitation, for each condition, a sample containing 500µl input chromatin but no antibody was also used. To ensure that all samples had been treated in a similar fashion during the ChIP experiment, H3 antibody was also incubated with input fragmented chromatin from each of the three conditions. As similar levels of H3 should be seen in all samples it can be used as a control to check that during the ChIP experiment all samples were treated similarly. A similar H3 pattern was observed in all 3 conditions (data not shown).

Real-time PCR using primers designed to locations in the *Hes1* promoter and coding region were used to detect the amount of DNA that was attached to Dmrt5. The regions to which the PCR primers were designed can be seen in Figure 4.8A. These are 2 binding sites in the promoter region (p0 and p2b), 1 non-binding site (p-2) and a binding site within the coding region (p4) (Figure 4.8A). To calculate if Dmrt5 binds to p0 and/or p2b (predicted binding sites) with a higher affinity than at non-binding sites, the binding at each site in samples incubated with Dmrt5 antibody was compared to the binding obtained for the same condition incubated with non specific antibodies to calculate the fold enrichment. As can be seen in figure 4.8B, at non-binding site p-2 there is a fold enrichment of 1 in all three conditions meaning that the non specific antibodies found in the serum and Dmrt5 antibody bind to this region with the same affinity. Therefore Dmrt5 does not bind to this region with greater specificity than a non specific antibody. Dmrt5 binds to the predicted binding sites p0 and p2b with a greater affinity than the non specific antibody. At p0 there is

enrichment in both *Dmrt5* over expression and wild type conditions. It is strange that there is a fold enrichment at this site in the Dmrt5 null condition too, as this should not contain any Dmrt5 protein and therefore the Dmrt5 null condition should give the same levels as the non specific antibody. At the p2b site a more expected profile is seen with over expression of Dmrt5 giving highest enrichment, followed by a lower level of enrichment in the wild type condition and no enrichment in the Dmrt5 null condition. In the p4 site, which is a predicted binding site in an exon of *Hes1*, only in over expressing cells does a specific binding occur. This is less than the enrichment seen at the two binding sites within the promoter. (Figure 4.8B).

Another way to analyse ChIP data is by comparing the amount of DNA pulled down relative to the starting material, the input, defined previously. This method is beneficial as one can see the relative levels of pull down for each condition (Figure 4.8C). The levels of pull down are much higher in the *Dmrt5* over expressing condition than in the other conditions, with levels of pull down in the null condition very low. The highest % input pull down is seen in p2b site.

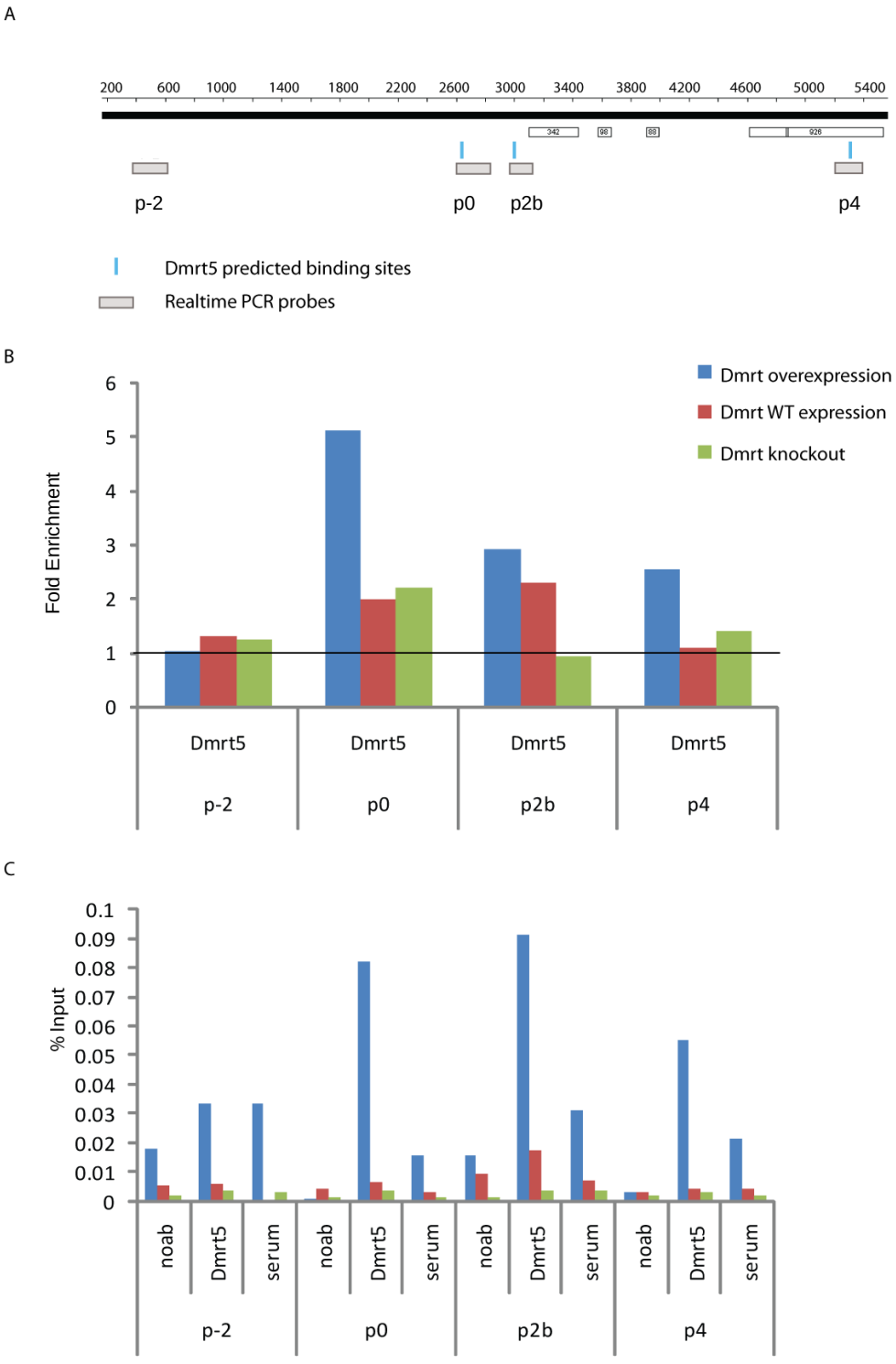


Figure 4.8. Analysis of Dmrt5 binding to *Hes1* promoter. A. Predicted Dmrt5 binding sites in *Hes1* coding region and upstream of the *Hes1* transcriptional start site (shown as blue lines). Grey boxes depict sites of PCR primers used. B. Real-time PCR of ChIP experiment at these probe sites calculated relative to non specific IgG

containing serum control. C. Real-time PCR of ChIP experiment at the described probe sites relative to % input.

4.2.4 Microarray analysis show Dmrt5 has a role in neurogenesis, proliferation and differentiation.

The experiments described in this chapter support the hypothesis that *Dmrt5* plays a role in neurogenesis of the ventral midbrain. To obtain a more global understanding of the function of *Dmrt5* during midbrain dopaminergic neuron development, I conducted a microarray experiment using HH st 16 ventral lateral midbrain tissues from embryos which had been electroporated with *pcaβ-IRES-GFP* or *pCaβ-Dmrt5-IRES-GFP* at HH st 10. The microarray experimental set up and analysis will be the topic of the next chapter. However, of relevance to this chapter and in agreement with the findings described above, tissue exposed to ectopic *Dmrt5* expression preferentially affected genes involved in CNS proliferation, CNS differentiation and neurogenesis, when compared to embryos exposed to *GFP* or electric current alone, as determined by Ingenuity Pathway Analysis (IPA) (Figure 4.9). The Ingenuity Knowledge Base is the largest knowledge base of its kind, with findings curated from the full text literature. IPA uses a Fisher's exact test to determine which biological functions were significantly enriched within the set of genes showing differential expression compared to the entire list of genes represented on the array.

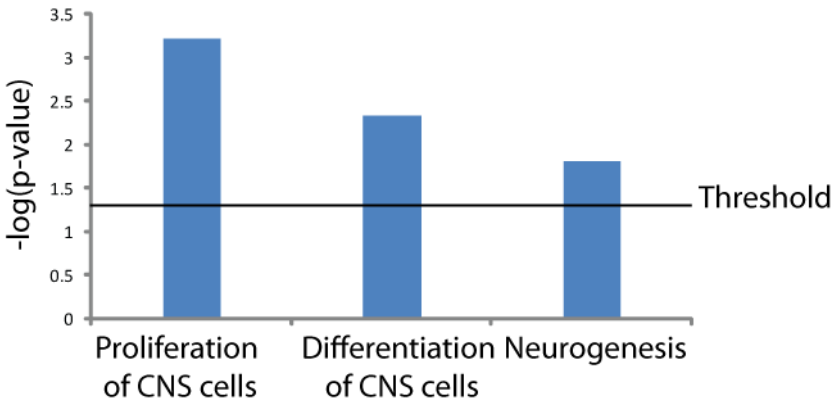


Figure 4.9. IPA analysis of microarray data showing that a significant number of genes involved in Proliferation of CNS cells, Differentiation of CNS cells and Neurogenesis are affected by over expression of Dmrt5 but not GFP.

4.3 Discussion

In this chapter I provide evidence that Dmrt5 regulates genes which are involved in ventral midbrain neurogenesis by altering expression of Tuj1, *Mash1*, *Ngn2*, *Hes1* and *Nato3* (Figure4.3). Dmrt5 is also involved in cell cycle progression, with over expression of *Dmrt5* causing G1 retardation of the cells and the change in expression of cell cycle genes involved in G1: *Cyclin D1* and *p27*, but no change in *p21*, S phase markers or G2/M checkpoint markers (Figure4.5 and 6).

Hes1 has only recently been investigated with regard to neurogenesis of the ventral midbrain [243, 244]. Here we show Dmrt5 binds to the promoter region of *Hes1* and can ectopically induce *Hes1* expression (Figure 4.7). Furthermore *Dmrt5* affects cell proliferation and neurogenesis; both of these processes are also affected by Hes1 [251]. As a result the effects observed upon *Dmrt5* over expression may be mediated via regulation of *Hes1* expression. Over expression of *Dmrt5* in cells where Hes1 is knocked down could be used to investigate if the neurogenic effect of Dmrt5 over expression is exclusively mediated via up regulation of *Hes1*.

Dmrt5 also causes the down regulation of *Nato3,* a gene known to repress *Hes1* expression and induce neurogenesis. Nato3 is proposed to permanently down regulate *Hes1* from E10.5 onwards [244]. However this contradicts results reported Lahti et al, which shows *Hes1* expression is still present at E11.5 [243]. It will be interesting to investigate the dynamics of Nato3, Dmrt5 and Hes1 expression during neurogenesis (E10.5-14) in more detail and specifically to investigate if they are expressed in the same cells. The creation of a good Dmrt5 antibody in 2011 should facilitate this process.

ChIP experiments show specific binding of Dmrt5 to two predicted binding sites in the *Hes1* promoter. Suggesting that *Hes1* is a direct target of Dmrt5, however these experiments must be repeated, both with biological replicates and preferably by a second method such as Electrophoretic shift mobility assays (EMSA).

During ventral midbrain development expression of ventral midbrain developmental genes, regulation of neurogenesis, cell cycle progression and exit are fundamentally linked [114, 117, 119, 244]. Dmrt5 appears to be involved in these processes. Dmrt5 affects neurogenesis and proliferation of the ventral midbrain, experiments in this chapter suggests that this is mediated, at least in part, via regulation of *Hes1* expression. These findings agree with those of Ross et al who previously postulated, based on their findings in C.elegans, that regulation of Hes genes by DM domain proteins may be a general mechanism [207]. Further experiments are required to better understand the way in which Dmrt5 is involved in ventral midbrain neurogenesis and regulation of cell cycle. Such experiments should include the analysis of mice homo or heterozygous for lack of Dmrt5.

Chapter 5. Genome-wide effect of Dmrt5 over expression

5.1 Introduction

In this chapter, I will use a genome-wide approach to investigate the possible functions of Dmrt5. It is important to interpret the results obtained from this experiment within the biological context of the ventral midbrain ventricular zone, as this is where *Dmrt5* is expressed during midbrain dopaminergic neuron development. Therefore, in the following paragraphs I will summarize the developmental processes occurring in this region from the start of *Dmrt5* expression at E9.5 (HH st 16).

When expression of *Dmrt5* first occurs, the ventral midbrain is entirely composed of a ventricular zone, containing proliferative progenitor cells, this remains the case until E10.5 (HH st 22) [107, 264]. From E9.5 (HH st 16) onwards cells start to express genes which commit the progenitors to dopaminergic fate such as Lmx1A and FoxA2 [115, 123, 124]. These specification factors also prevent expression of genes involved in more lateral fates [123] and activate expression of proneural gene Ngn2 which is concomitant with cell cycle exit [115]. As this occurs the cells transition from the ventricular zone to form a new layer, the intermediate zone, containing immature dopamine neurons. These immature neurons activate transcription of genes involved in terminal differentiation such as Nurr1 [147, 265, 266] and Pitx3 [156, 157]. The terminally differentiated cells are located in a third layer, the marginal zone, and express TH and other components required for dopamine neurotransmission. This process is described in more detail in Chapter 1 and Figure 1.4.

The cells of the ventricular zone give rise to midbrain dopaminergic neurons continuously from E10.5 to E14.5 (HH st 22 – HH st 34) as determined by expression of TH [103, 106]. During the continuous generation of dopamine neurons, cell proliferation only occurs in the ventricular zone, even after the formation of the three layers [103, 106]. It has been estimated that the number of cells present in the ventral midbrain of a laboratory mouse increases about 10 fold from E10.5 to E12.5 from 1-2x10^{e4} to 10-20 x10^{e4} [264]. As such the ventricular zone provides the progenitor population and the proliferative capacity of this region which is crucial to the generation of the full complement of dopamine neurons.

How cells transition from the ventricular zone to the intermediate zone is not well understood. It has been shown that cells on the ventricular surface of the ventral midbrain as labelled by BrdU progress ventrally from this ventricular zone to the marginal zone where they express TH. Electron microscopy showed that the immature neurons made close contacts with radial glia cells [267]. This led to the perception that the radial glia like cells serve as a scaffold for dopamine neuron migration [267, 268] although this has not been experimentally determined. Further studies have shown that cells expressing radial glial markers can undergo neurogenesis and give rise to dopaminergic neurons [106].

Since each layer of the developing neural tube contains cells in different stages of differentiation, progression through the layers may be integrally linked to the developmental program of dopaminergic neuron differentiation. However there is little research to investigate this. Loss of Ngn2, required for neurogenesis of midbrain dopaminergic neurons, causes cells in the ventral midbrain to remain attached to the apical surface, with a decrease in number of cells in the marginal zone [114, 117] providing evidence that the transition of cells from one layer to the next is linked to

the state of the progenitor cell. In support of this, conditional knock out of the cadherin-dependent cell adhesion complex, β-catenin [269] which is expressed abundantly at the apical side of the ventricular zone, in midbrain dopaminergic progenitor cells resulted in severe perturbation in the organization of ventral midbrain floor plate and a reduction in cell proliferation and neurogenesis [177].

In addition to the specific role of the ventricular zone, floor plate cells play an important role in guidance of commissural axons by secreting diffusible molecules such as netrins [270, 271].

To investigate if Dmrt5 is involved in any of these processes which occur in the ventral midbrain a microarray experiment was carried out. The aim of the microarray experiment was twofold:

1. To determine if Dmrt5 is involved in patterning of the ventral midbrain, as many other transcription factors expressed in ventricular zone promote expression of downstream dopaminergic factors and inhibit expression of lateral gene fates [119, 123].

2. To identify genes regulated by Dmrt5 to infer other possible functions of this gene in the ventral midbrain development.

To achieve these goals the genome-wide expression profile of Ventral Lateral Midbrain (VLM) tissue from HH st 16 (E9.5) chicken embryos exogenously expressing *Dmrt5* was compared to the expression profiles of stage matched controls and to the expression profile of floor plate (FP) tissue, a region in which *Dmrt5* is normally expressed and which gives rise to dopaminergic neurons. We

reasoned that exogenous expression lateral to the normal expression region would identify genes that were directly regulated by the transcription factor Dmrt5 and pathways downstream of this. Furthermore comparison of the transcriptional profile obtained upon *Dmrt5* over expression in the VLM to the expression pattern normally seen in the FP would provide information regarding the role of Dmrt5 in patterning of the ventral midbrain.

5.2 Results

5.2.1 Experimental strategy

The experimental design can be seen in Figure 5.1. Pooled samples of VLM tissue and a pooled sample of FP tissue were collected. The five samples were:

Control Condition

1. Unelectroporated VLM (**VLM**)

Experimental Conditions

2. VLM exposed to electric current only (**VLMi**)
3. VLM exposed to expression of *GFP* and electric current (**VLMg**)
4. VLM electroporated with *Dmrt5* (**VLMd**)
5. FP from unelectroporated embryos (**FP**)

Each pooled sample contained tissue from 6 stage matched embryos. Exogenous expression was achieved by *in ovo* electroporation of *pCAβ*-Dmrt5-*IRES-GFP* or *pCAβ-IRES-GFP* at HH st10. Embryos were collected 24 hours later at HH st16.

Following collection of tissue, RNA was extracted, amplified using Ribo-SPIA technology which allows linear, isothermal amplification of the mRNA species in a total RNA population. Unlike exponential amplification, this linear amplification approach is carried out by replication of only the original transcripts not replication of copies, resulting in a high-fidelity, robust and sensitive amplification process that reflects the original RNA sample. This amplified RNA was then hybridised to Affymetrix GeneChip Chicken Genome Microarrays containing probes for 32,773 transcripts corresponding to over 28,000 genes. For each condition three biological replicates were used.

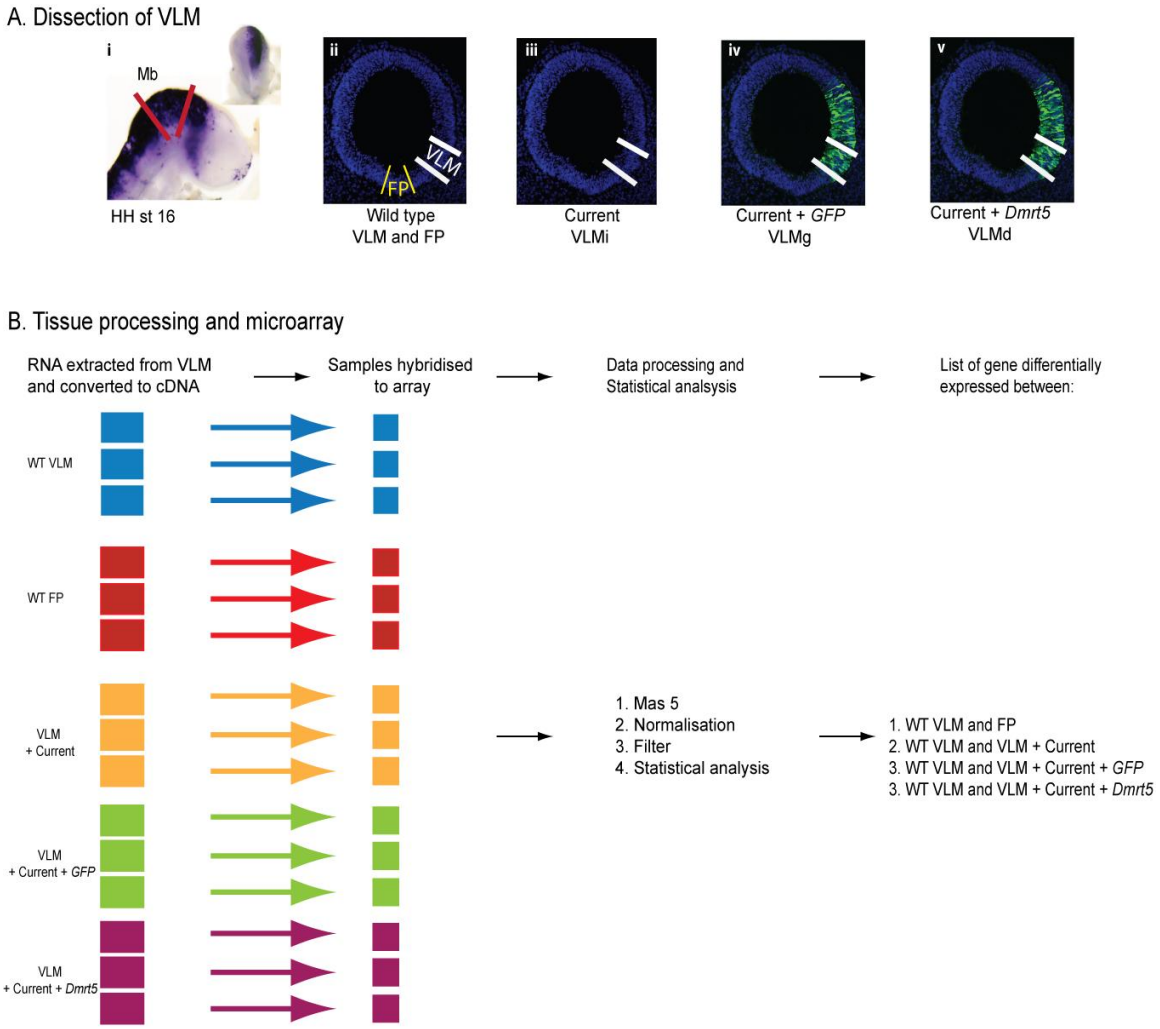


Figure 5.1. Experimental strategy A. Dissection of the ventral lateral midbrain (VLM) and Floor plate (FP) region. (i) *In situ* sagital view of a HH st 16 electroporated embryo expressing *pCAβ-IRES-Dmrt5* construct (dorsal view in the inset). Red lines indicate the midbrain region (Mb), which was dissected out. (ii-iv) Coronal sections of midbrain. White lines mark the VLM regions and yellow lines indicate the FP region isolated for microarray analysis. The regions isolated for microarray analysis were (ii) control embryos wild type VLM (VLM) and wild type floor plate (FP), (iii) VLM exposed to current (VLMi), (iv) VLM exposed to current + *GFP* (VLMg), and VLM exposed to current + *Dmrt5* (VLMd; image not shown). **B.** Tissue processing and microarray analysis. Six VLM tissues were pooled for each biological replicate, and three biological replicates were used for each condition. cDNA was isolated from these pools and hybridised to the Affymetrix Chicken Genome Array. Following Mas5, normalization and filtering, genes whose expression differed significantly between the wild type VLM and FP, VLMi, VLMg and VLMd were identified by One-way ANOVA, SNK post hoc test and fold change analysis.

5.2.2 Data processing - Validation and filtering of microarray data

The hybridisation of labelled samples to the probes on the Affymetrix GeneChip Chicken microarrays was calculated from raw data using the Mas5 algorithm embedded into the GCOS suite (Version 1.2 Affymetrix). Since many of the genes involved in midbrain development are transcription factors, Mas5 was chosen as the other commonly used algorithm, RMA, squashes the ends of the data sets so information about low expressing genes such as transcription factors would not be so easily identifiable [272]. Data were then analysed using the Genespring package (version GX11; Aglient technologies).

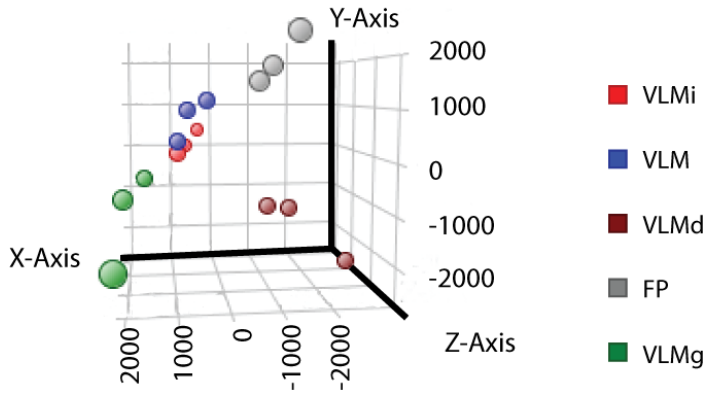
The suitability of the expression data sets for inclusion in further analysis was assessed using three methods. First the 3'/5' ratio, this measures the efficiency of cDNA synthesis reaction. All Affymetrix arrays contain probes for the regions corresponding to the 3', middle and 5' of housekeeping genes such as GAPDH and β-Actin. The ratio of signal intensity of 3' and 5' probe sets provides a measure of the number of cDNA synthesis reactions that went to completion, good quality RNA should result in a ratio of 5':3' = 1. A 5':3' of greater than 3 indicates that either the starting RNA was degraded or there were problems with the cDNA synthesis reaction. All 5':3' ratios were around 1 and none were 3 or higher. This indicates that the RNA was of good quality and the cDNA synthesis reaction occurred correctly.

Second the hybridisation controls were analysed. Premixed hybridisation control transcripts in known staggered concentrations were added to the hybridisation mix. Hybridisation controls are composed of a mixture of biotin labelled cRNA transcripts of bioB, C and D and Cre prepared in staggered concentrations. These controls enable monitoring of the hybridisation and washing process. The signal intensity should increase with concentration. Deviations from the expected intensity profile of these controls indicate potential problems with the hybridisation or washing process. All samples showed similar and expected profiles of hybridisation.

Lastly the variation in biological replicates was analysed by Principal Components Analysis (PCA). PCA is a mathematical procedure that measures the variation in expression levels between microarrays. PCA showed all biological replicates cluster together, demonstrating that the biological replicate datasets are similar and reproducible (Figure 5.2A).

The overall relationship between biological replicates was also assessed using quantile plots for each of the 15 microarray data sets. The distribution of gene expression across the whole array was monitored before and after normalization by quantile groupings and was found to be similar for each set of biological replicates. The quantile plots for the normalized values can be seen in Figure 5.2B.

A



B

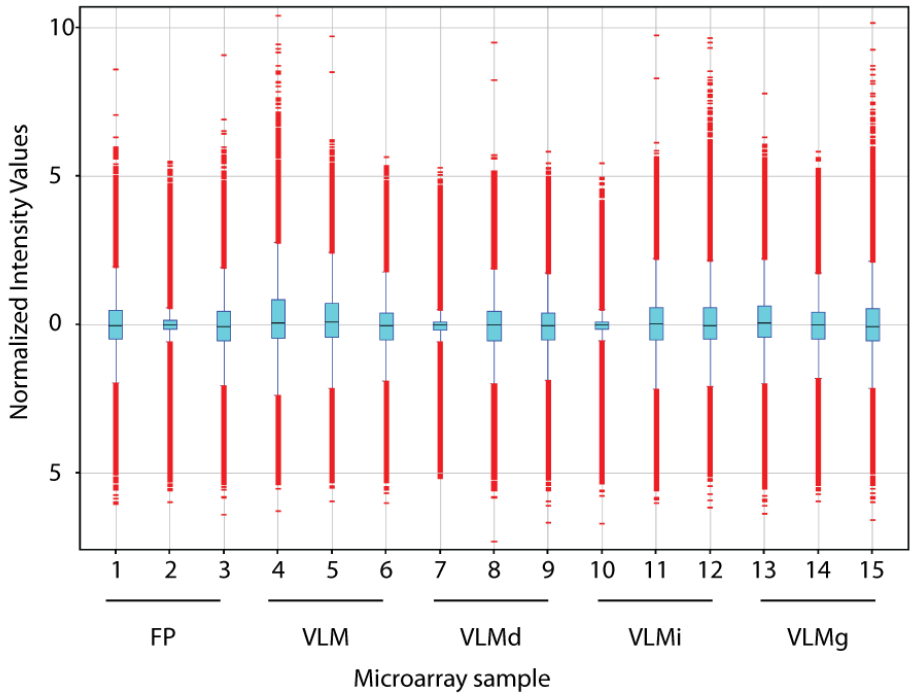


Figure 5.2 Validation of microarray data. A. PCA analysis. Each dot represents the expression profile from one microarray. Dots are colour coded into biological replicates. All biological replicates cluster together, indicating that biological replicates are similar. **B.** Quantile box plot of normalised data for each microarray. The blue box shows the 25th to 75th percentile, the band in the middle the 50th percentile of the normalised intensity values from each microarray. The end of the whiskers shown in red represents one and a half standard deviations from the mean.

Having confirmed that all samples passed quality control, probe sets showing acceptable values in all 3 biological replicates of any 1 of the 5 conditions were then selected. These probe sets were analysed to identify genes significantly differentially expressed between the control condition VLM and each experimental conditions. This was done using One Way Analysis Of Variance (ANOVA) with unequal variance. One-way ANOVA tests allow one to determine if there is a significant effect on gene expression behaviour across any of the groups under study relative to the control condition. A significant p-value resulting from a one-way ANOVA test would indicate that a gene is differentially expressed in at least one of the groups analysed. If there are more than two groups being analysed, however, the one-way ANOVA does not specifically indicate which pair of groups exhibits statistical differences. Post Hoc tests can be applied in this situation to determine which specific pair or pairs show significant differential expression.

The Post Hoc test chosen was Student-Newman-Keuls (SNK) test. In this test all means for each condition are ranked in order of magnitude, with the group with lowest mean gets a ranking of 1. The pairwise differences between means, starting with the largest mean compared to the smallest mean, are tabulated between each group pair and divided by the standard error. This value, *q,* is compared to a critical value. If *q* is larger than the critical value, then the expression between that group pair is considered to be statistically different. The SNK test is slightly less conservative than the Turkey test, the other common Post Hoc test, and this is why it was chosen. Multiple testing correction is a way to remove false positives from the list of differentially expressed genes, however it is often too stringent for biological tests therefore it was not used.

Of these genes showing a significant differential expression ($p < 0.05$) those with a ≥1.5 fold change from the level in the control condition relative to an experimental control were selected. The number of probe sets differentially expressed between each experimental condition and the control condition can be seen in Table 5.1.

Experimental condition	**Number of probe sets showing a significant differential expression relative to the control condition (VLM) ($p < 0.05$, fold change ≥1.5)**
VLMd	1783
VLMg	555
VLMi	450
FP	1381

Table 5.1. Number of probe sets showing significant differential expression ($p < 0.05$) and ≥ 1.5 fold change between experimental condition and control condition VLM.

Any probe sets showing a change in expression upon exposure to current (VLMi) or GFP (VLMg) were removed from further analysis (Figure 5.3), as these changes could be an artefact of the electroporation technique. Following removal of these probe sets 1545 probe sets were differentially expressed between VLMd and control VLM. This included 296 down regulated and 1229 up regulated probe sets.

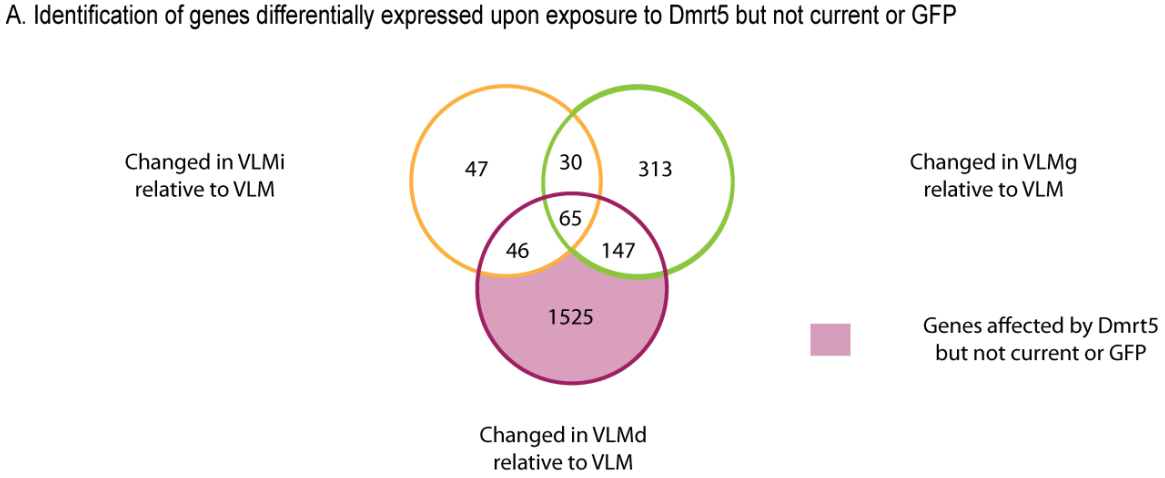


Figure 5.3. Identification of probe sets significantly differentially expressed (p <0.05) and showing a ≥1.5 fold change in VLMd relative to VLM but not affected in VLMg or VLMi relative to VLM.

5.2.3 Dmrt5 role in floor plate transcription

In Figure 5.4 the normalized intensity value for each probe set significantly differentially expressed ($p < 0.05$, fold change ≥ 1.5) between the VLMd and VLM is represented as a line. The lines are colour coded based on fold change from VLMd to VLM with red indicating up regulation and blue down regulation. Exogenous expression of *Dmrt5* causes a large number of genes to display an expression profile similar to that in the FP. This led to the hypothesis that Dmrt5 may induce a transcriptional profile similar to that of the floor plate, the region from which dopaminergic neurons arise and the region where *Dmrt5* is endogenously expressed (Figure 5.4).

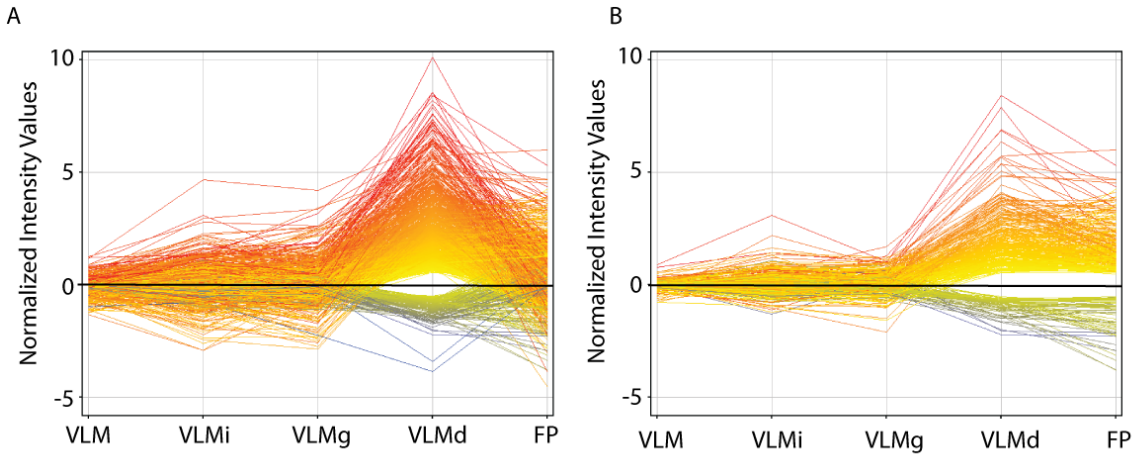


Figure 5.4. Profile plots. **A.** Profile plot of probe sets differentially expressed between the VLMd and VLM. **B.** Profile plot of probe sets differentially expressed between the VLMd and VLM which show a similar expression profile to the FP.

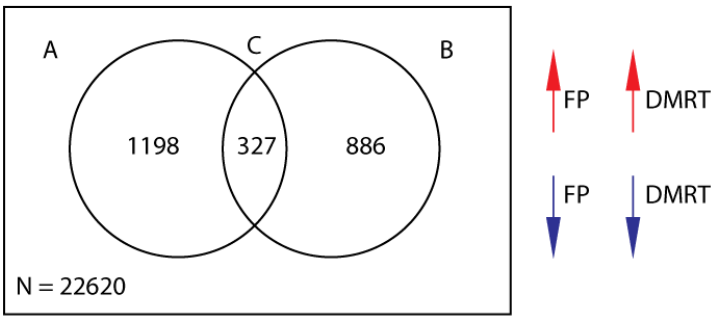
To investigate this hypothesis further we identified probe sets which were up regulated in both the VLMd and FP conditions, and genes down regulated in both VLMd and FP conditions relative to the VLM. As the null hypothesis I carried out the opposite comparison of genes which were up regulated in the VLM and VLMd conditions, and genes down regulated in VLMd and VLM conditions. These findings are summarised in Table 5.2.

	Up in FP	Down in FP
Up in Dmrt5	266	31
Down in Dmrt5	55	61

Table5. 2. Probe sets differentially expressed in VLMd relative to VLM, divided into up and down regulated FP genes.

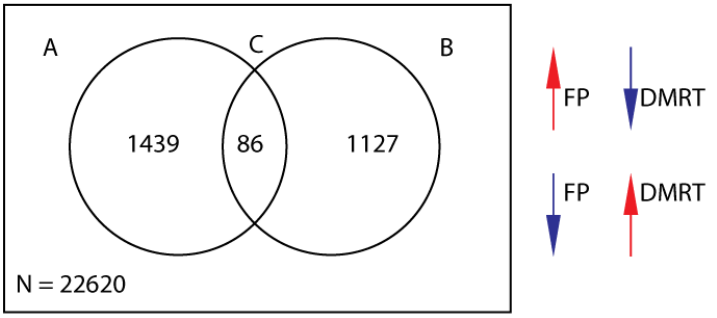
Hypergeometric testing was carried out to see if the trend observed in the expression profile plots was statistically significant. This was calculated using the phyper function in R. The trend that Dmrt5 exogenous expression in the VLM induces a FP like character is significant ($p=5^{e-116}$) whilst the opposite hypothesis has a p=0.27 and therefore is not statistically significant (Figure 5.5).

A. Evidence in support of hypothesis Dmrt5 induces a floor plate like character



p=5e-116

B. Evidence in support of hypothesis Dmrt5 induces a ventral lateral like character



p=0.27

A = Dmrt5 regulated probe sets (1525)
B = Probes sets differentially expressed between FP and VLM tissues (1213)
C = Number of prob sets overlaping between groups A and B
N= Total number of entities on array present in any on of the experimental conditions (22620)

Hypergeometric equation :

$$P(X=C) = \frac{\binom{B}{C}\binom{N-B}{A-C}}{\binom{N}{A}}$$

R script used : phyper = (C, A, N-A,B) lower.tail = F

Figure 5.5. Hypergeometric testing of the hypothesis that Dmrt5 induces a FP like transcriptional profile. A. Evidence in support of this hypothesis **B.** Evidence in support of the opposite hypothesis that Dmrt5 induces a transcriptional profile similar to that of the VLM**.**

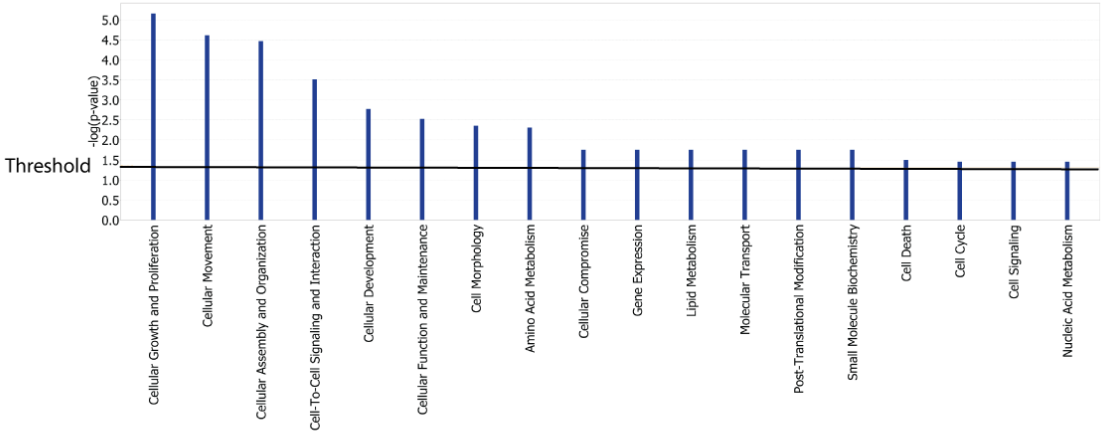
5.2.4 Analysis of genes differentially expressed in VLMd and FP relative to VLM

To determine the function of genes regulated by Dmrt5 and normally found in the FP, this probe set list was analysed using Ingenuity Pathway Analysis (IPA) v8.0-2602. The Ingenuity Knowledge Base is the largest knowledge base of its kind, with findings curated from the full text literature. For analysis, the Affymetrix Chicken Genome Array gene list was used as a reference set. All data sources, all species, and all nervous system tissues and cells were used for the analysis. IPA uses a Fisher's exact test to determine which biological functions were significantly enriched within the set of genes showing differential expression compared to the entire list of genes represented on the array. One reason for choosing IPA was that this analysis allows selection of the Chicken genome as a reference set from which to identify significantly enriched categories.

The biological categories of the genes differentially expressed in the VLMd and FP relative to the VLM can be seen in Figure 5.6A.The most significantly affected molecular and cellular function is “Cellular growth and proliferation”. The second most common function is “Cellular movement” within this category the genes listed are involved in migration of eukaryotic cells, migration of brain cells and guidance of axons. Within “Cellular assembly and organization”, growth of neurites is the most affected function. Within the category “Cell-cell signalling and interaction” the majority of genes have a role in adhesion of cells. Whilst within the category “Cellular development” most genes are involved in differentiation of neurons. In accordance with the findings that Dmrt5 up regulates Hes1 identified in Chapter 4; Hes1 is found by this analysis as normally present in the FP and ectopically induced in the VLMd relative to VLM. In agreement with the findings that genes involved in cellular

movement, cell adhesion and neurite growth are affected in the VLMd and FP changed gene list, “Axonal guidance” and “Actin cytoskeleton” canonical pathways are significantly enriched (Figure 5.6B).

A. Molecular and Cellular Functions enriched in the genes changed in VLMd and FP relative to VLM



B. Canonical Pathways enriched in the genes changed in VLMd and FP relative to VLM

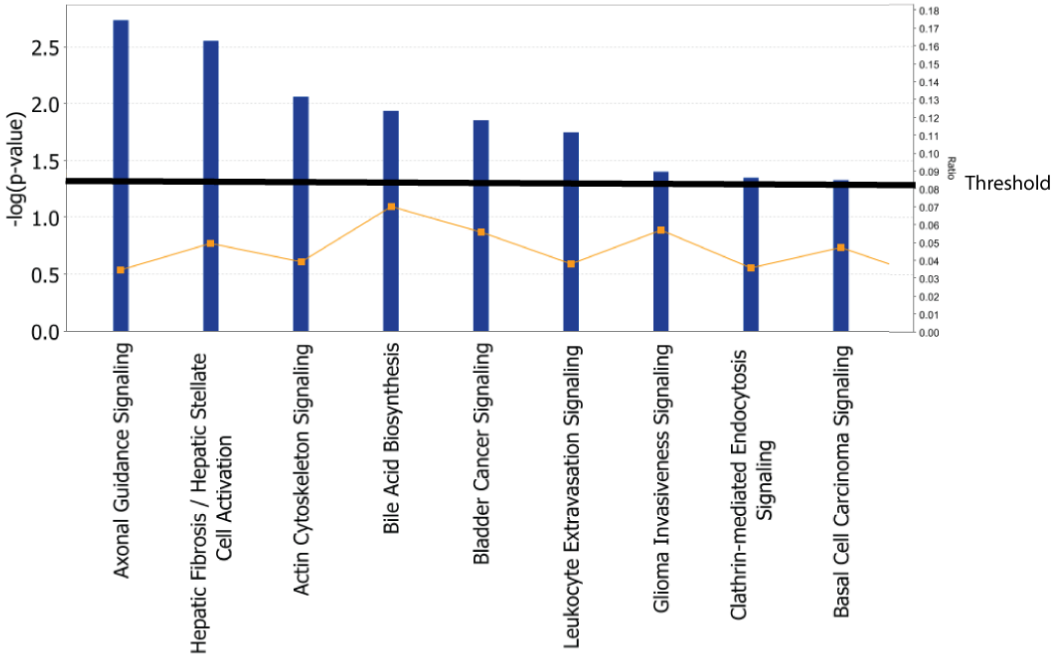


Figure 5.6. Analysis of the list of genes with the same fold change trends in VLMd and FP relative to VLM A. Molecular and cellular functions associated with this gene list using IPA. **B.** Canonical pathways associated with this list of genes using IPA. The significance of the association between the data set and functions or canonical pathway was measured by a Fischer's exact test. This calculates a p-value determining the probability that the association between the genes in the data set and the function or pathway is explained by chance alone, a p value of < 0.05 is considered significant and is marked by the threshold line. For canonical pathway analysis a second method to determine significance is also carried out this is the ratio of the number of differentially expressed transcripts from the data set that map to the pathway divided by the total number of molecules that exist in the canonical pathway (right y axis).

Gene	Fold change	Gene	Fold change
UNC13C	-4.60	DLK1	-1.81
ALDH1A3	-3.92	ADAMTS6	-1.80
C13orf15	-2.99	CABLES1	-1.78
PCNX	-2.95	MTHFD1	-1.75
IL17RD	-2.71	PTPRZ1	-1.74
SPRY2	-2.68	RAD54L	-1.73
LOC421644	-2.59	CPSF2	-1.71
RASL11B	-2.50	ZFP106	-1.69
FGF18	-2.41	GTPBP5	-1.69
DIO3	-2.39	LIPA	-1.68
PITPNC1	-2.37	FGF13	-1.68
FGF3	-2.34	METT5D1	-1.65
PLCH1	-2.15	EPHB6	-1.59
MEIS2	-2.15	ARRDC4	-1.59
VIP	-2.10	LOC423506	-1.58
ELMOD1	-2.01	GLI3	-1.58
C6orf163	-1.95	SIPA1L1	-1.58
USP44	-1.94	SSB	-1.56
ST3GAL5	-1.93	ZNF516	-1.56
CEP152	-1.91	WNT7A	-1.56
RAB3IP	-1.88	TYW3	-1.54
MAPKAP1	-1.87	SLC24A5	-1.51
TPH2	-1.83	SOAT1	-1.50

Table 5.3.Genes showing down regulation (≥ 1.5 fold change, p <0.05) upon exogenous expression of Dmrt5 in the VLM and which are down regulated (≥ 1.5 fold change, p <0.05) in the FP relative to VLM.

Gene	Fold Change	Gene	Fold Change	Gene	Fold Change	Gene	Fold Change
S100A6	216.22	AKAP 14	8.07	ITGB5	3.51	FSTL4	2.12
LOC3951 92	203.34	COL6 A1	7.96	LOC768 772	3.51	SEMA 3F	2.09
LOC4278 38	137.35	IGSF1 1	6.63	ST14	3.51	SNTB 1	2.04
WFDC2	67.73	CITED 4	6.45	SDK2	3.48	PACS 2	2.04
FABP3	64.70	LINGO 3	6.42	LOC425 593	3.44	BTBD 11	2.03
LCAT	56.24	GLUL	6.23	KIF9	3.33	CCDC 91	2.01
ATP2A3	55.31	RAB8 B	6.16	PTGDS	3.28	RCJM B04_9 b22	2.01
CFI	42.45	CXorf3 6	5.99	KRT19	3.27	IGFBP 7	2.01
CRYBA2	40.92	GPSM 1	5.87	NHSL2	3.25	C10orf 63	1.98
THPO	26.28	RSPH 1	5.62	PCMTD 1	3.22	C9orf7 5	1.97
RPRML	24.46	TNS1	5.44	NME1	3.18	DUSP 14	1.97
CALB2	22.75	KIAA1 529	5.32	EDG4	3.12	OTOR	1.94
LOC4308 80	18.04	LOC41 6899	5.26	IGFBP2	3.09	CLDN 11	1.93
ACE	17.34	SARD H	5.16	NFRKB	3.07	NPDC 1	1.91
CNRIP1	16.23	YPEL2	5.12	DCN	3.05	FOXA 2	1.87
PPL	15.88	RCJM B04_3 a16	4.95	LOC417 151	3.01	RNF12 8	1.85
RASD1	14.61	VDAC 1	4.87	WDR52	2.96	COL5 A1	1.85
PNAT10	14.39	CALD 1	4.82	FRMD4 B	2.93	BCAR 1	1.84
RTN4R	13.59	ITGB1 BP2	4.65	FGF12	2.85	CLCN 7	1.83
PDGFB	13.58	KDR	4.62	SLC9A3 R1	2.85	PELI2	1.82

TMEM51	13.57	RAB26	4.61	NMI	2.81	PGM3	1.81
TUBA4A	13.55	CXCL12	4.58	JDP2	2.81	TXNDC16	1.80
SCG2	13.25	**HES1**	**4.54**	PCDP1	2.72	FN1	1.80
CHRD	12.99	S100A11	4.43	CDKL2	2.68	VTN	1.79
LOC772356	12.79	TMEM63C	4.31	CREG1	2.60	PSAP	1.78
PLP1	11.06	FAM20A	4.29	DNAI2	2.57	DAP	1.78
LOC425403	11.02	UNC5B	4.24	LOC424678	2.56	CTHRC1	1.76
FAM101B	10.71	RCJMB04_1d13	4.17	LOC424161	2.54	NCAM1	1.73
MMP28	10.70	EPB41L3	4.12	FZD4	2.47	IFNAR2	1.72
STK10	10.52	IGFBP5	4.08	OLFM1	2.44	PEX11G	1.69
RAPGEF3	10.27	CADM1	3.98	RCJMB04_6f1	2.43	ZDHHC23	1.68
GDPD5	10.09	CREB3L2	3.93	DNAI1	2.42	SLC16A2	1.63
PLXNA2	9.99	ARHGEF9	3.93	SLC16A6	2.40	LRRC48	1.62
LTF	9.86	EFEMP1	3.88	MYL9	2.33	CPE	1.58
DYDC1	8.96	LOC395611	3.82	ANXA2	2.32	SLC17A5	1.57
COL9A1	8.68	ANXA6	3.63	LOC420008	2.26	GGTL3	1.56
OLFML3	8.56	RHOJ	3.59	STYK1	2.26	JAZF1	1.55
DLC1	8.19	ARRDC1	3.57	LOC422229	2.21	ENTPD2	1.53

Table 5.4.Genes showing up regulation (≥ 1.5 fold change, p <0.05) upon exogenous expression of Dmrt5 in the VLM and which are up regulated (≥ 1.5 fold change, p <0.05) in the FP relative to VLM. Hes1 is shown in Bold.

5.2.5 Genes differentially expressed upon exogenous expression of Dmrt5.

At the time of tissue collection for the microarray, expression of *Dmrt5* is only just initiated in the most anterior region of the ventral midbrain. Therefore the endogenous functions of Dmrt5 in the floor plate (FP) may be missed by analysis of genes that are differentially expressed both upon exogenous *Dmrt5* expression in the VLM and in the FP relative to the VLM at HH st16. To identify other genes regulated by Dmrt5 which may be involved in later midbrain dopaminergic neuron development, all genes significantly differentially expressed ($p < 0.05$, fold change ≥ 1.5) in the VLMd condition relative to VLM were analysed by IPA.

As was the case in the genes set showing a fold change in both the FP and VLMd relative to VLM in the previous section, and in accord with the findings described in Chapter 4, “Cell growth and proliferation” was the most significantly enriched biological category with 32 molecules. The category “Cell movement” contains 38 molecules; within this category most of the genes are involved in migration of cells and guidance of axons. Within the 3rd most significantly enriched function, “Cell-cell signalling and interaction”, 18 molecules involved in attachment and adhesion were affected. The category “Cellular development” contains 40 molecules most of which have been cited to effect differentiation of neuronal cell lines or *in vivo* are involved in differentiation of central nervous system tissue. “Gene expression” and “Lipid metabolism” were also significantly affected, as was “Cell death”. Figure 5.7A.

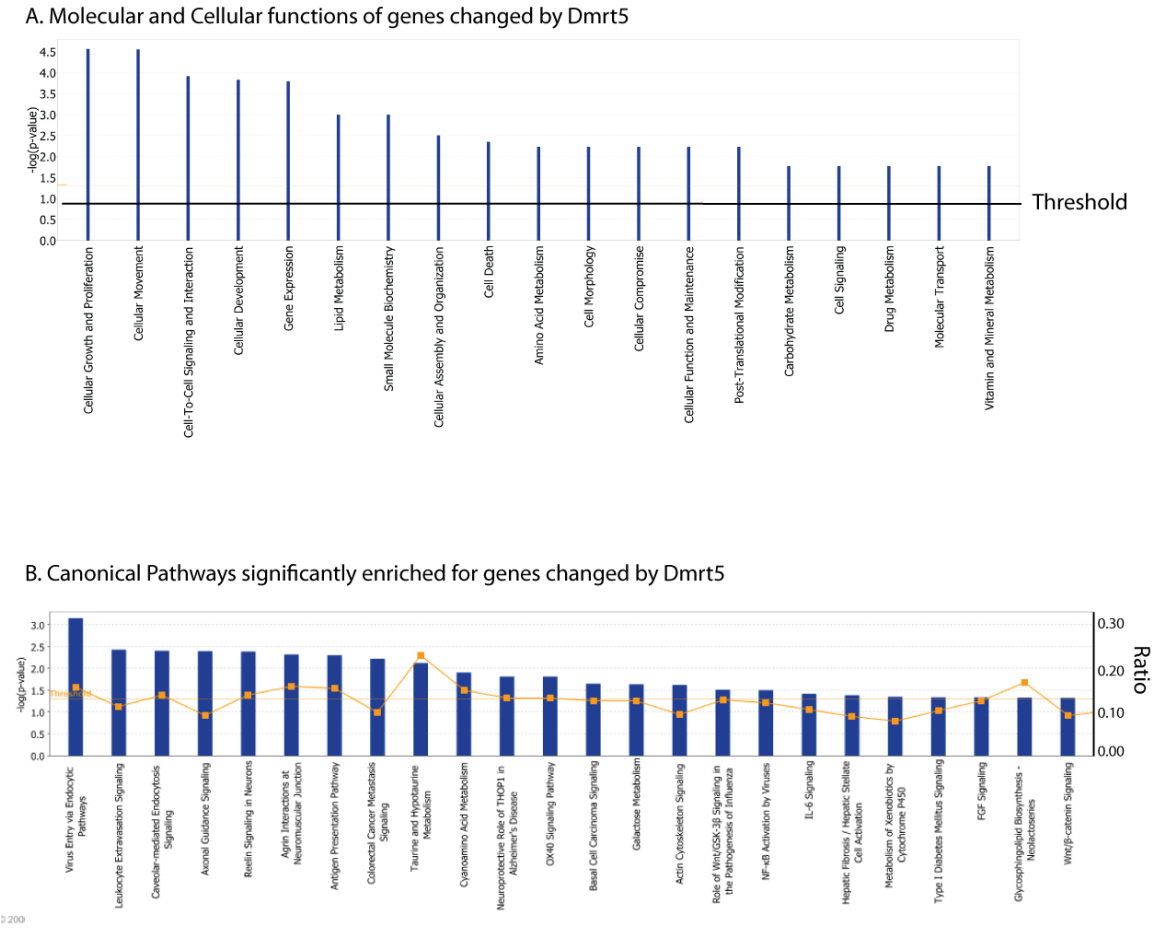


Figure 5.7. Analysis of the list of genes with fold change in VLMd relative to VLM.
A. Molecular and cellular functions enriched for in the list of genes significantly differentially expressed in the VLMd relative to VLM.**B.** Canonical Pathways significantly enriched for in the genes showing significant differential expression in the VLMd relative to VLM condition. The threshold shows p= 0.05, therefore any bars above this line are categories which are significantly enriched. The ratio (B) (right y-axis) is the number of differentially expressed probe sets from the data set that map to the pathway divided by the total number of molecules that exist in that pathway.

Analysis of the canonical pathways significantly enriched in the Dmrt5 gene set revealed that genes regulated by Dmrt5 which can be directly linked to a function during midbrain dopaminergic development are “Axonal Guidance signalling” (the 4th most significant pathway) and “Reelin signalling in neurons” (the 5th most significant pathway). The most enriched pathway was “Virus entry via endocytic pathway”,

“Leukocyte extravasion signalling” and “Clatherin-mediated endocytosis signalling”. “FGF signalling” and “Wnt/beta catenin signalling” were also slightly but significantly enriched.

5.2.6 Validation of the microarray by real-time PCR of a selection of candidate genes.

Microarray analysis and subsequent IPA analysis of the data sets identified that the genes affected by exogenous expression of Dmrt5 in the VLM are involved in the functional categories “Cell growth and proliferation”, “Cellular movement”, which largely consists of genes involved in cell migration and axon guidance. “Cell-cell signalling and interaction” is also significantly enriched, with most of the genes involved in this function cited as involved in attachment and adhesion. “Cellular development” was also a significantly enriched category within which genes involved in differentiation of neurons are the most common. Furthermore, comparison of genes differentially expressed between VLMd and VLM and FP and VLM respectively showed that Dmrt5 exogenous expression in the VLM promotes a transcriptional profile that is statistically significantly similar to that of the FP.

To validate these findings, the expression of genes involved in these categories were analysed in VLM, FP, VLMd and VLMg samples by real-time PCR. All candidate genes selected were chosen from the genes significantly differentially affected in the same way in both VLMd and FP relative to VLM. By selecting this set of genes the finding that *Dmrt5* exogenous expression in the VLM induces a transcriptional profile similar to the FP could also be validated by real-time PCR. These can be seen in Figure 5.8.

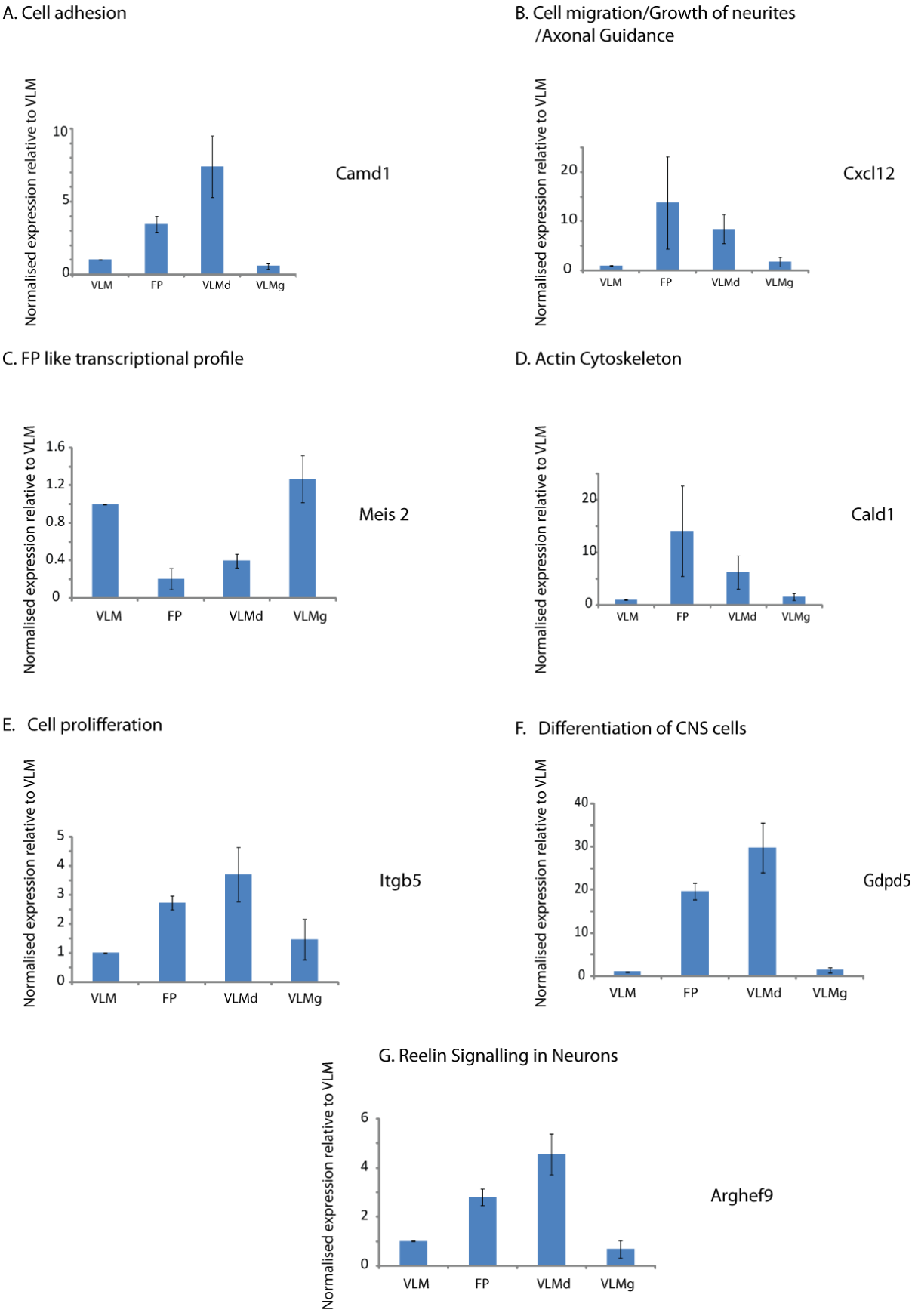


Figure 5.8. Validation of microarray and IPA analysis. Real-time PCR of genes involved in each of the major categories significantly affected by exogenous expression of *Dmrt5* in

the VLM. Expression is relative to expression in the VLM, each bar graph is an average of three biological replicates, and each biological replicate contained tissue from 6 stage matched embryos. Error bars show standard deviation.

No previous publications have described the role of Dmrt5 or members of this gene family in cell adhesion and cell migration. However, in agreement with these findings from the microarray, *in ovo* electroporation of Dmrt5 into the midbrain does disturb the cellular morphology (Figure 5.9). As can be seen in Figure 5.9 midbrain neuroepithelial cells overexpressing GFP at HH st 16 attach to the apical and basal laminar creating long cells spanning the width of the epithelium. However upon overexpression of Dmrt5 cells no longer have this linear morphology and are round with some cells having no attachment to either the apical or basal laminar.

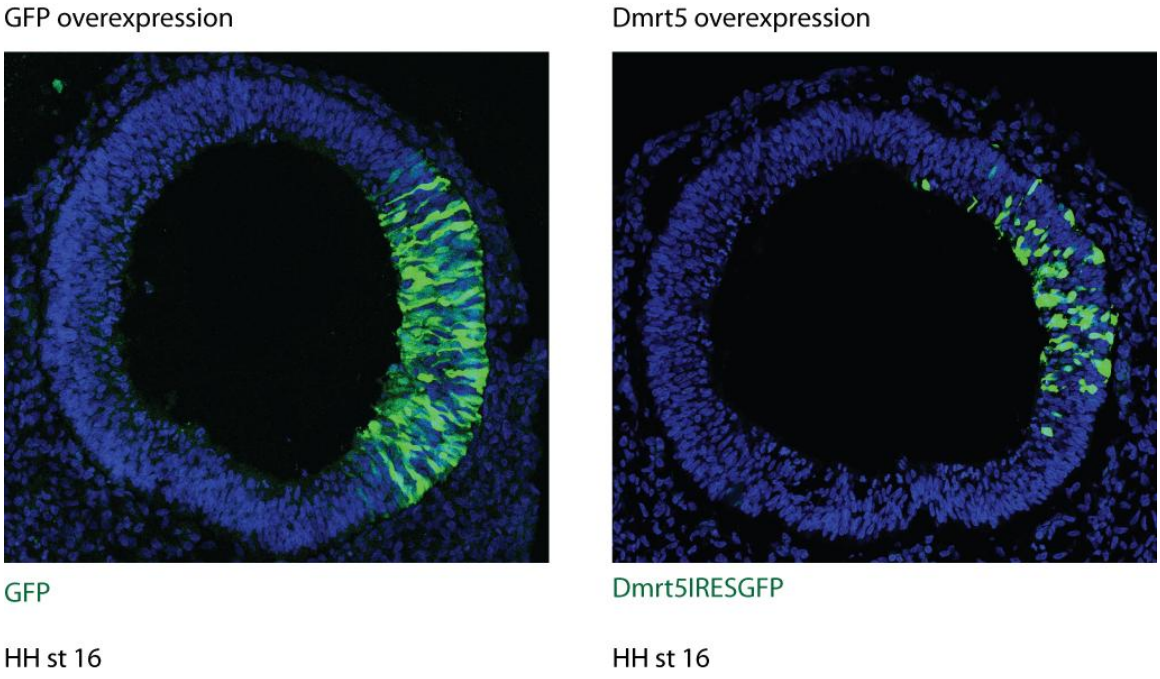


Figure 5.9. Coronal Sections of HH st16 midbrains electroporated at HH st10 with (A) *pCAβ-IRES-GFP* or (B) *pCAβ-Dmrt5-IRES-GFP.* Sections are stained with DAPI to show nuclei (blue); green is expression of GFP from electroporated construct. Cells over expressing *Dmrt5* **(B)** display an unusual morphology.

5.2.7 Identification of novel genes expressed in the floor plate

Comparison of the transcriptional profile of the VLM and FP at HH 16 provides a snap shot of the gene expression present in the ventral midbrain just after the midbrain region has been defined as and such provides information about the gene expression profile of the ventral midbrain as specification of midbrain dopaminergic neurons is initiated. Therefore the genes up regulated in the FP relative to the VLM may lead to a better understanding of the developmental program involved in formation of midbrain dopaminergic neurons. To look at the functions in which these genes are involved, the genes differentially expressed in the floor plate and VLM were analysed by IPA.

The top cellular and molecular functions significantly enriched for in this data set are “Cellular movement”, “Cell growth and proliferation”, “Cellular assembly and organisation” and “Cell development”. Interestingly “Cell death” is the 5th most significant cellular and molecular function in normal floor plate cells. The canonical pathways affected are shown in figure 5.10. “Axonal guidance” is the most affected category, followed by several which have no function in development of the midbrain such as “Hepatic fibrosis”, “Basal cell carcinoma signalling” and “Atherosclerosis”. “Human embryonic stem cell pluripotency” is the 8th most significant category. “Wnt/beta catenin signalling” is also significantly enriched.

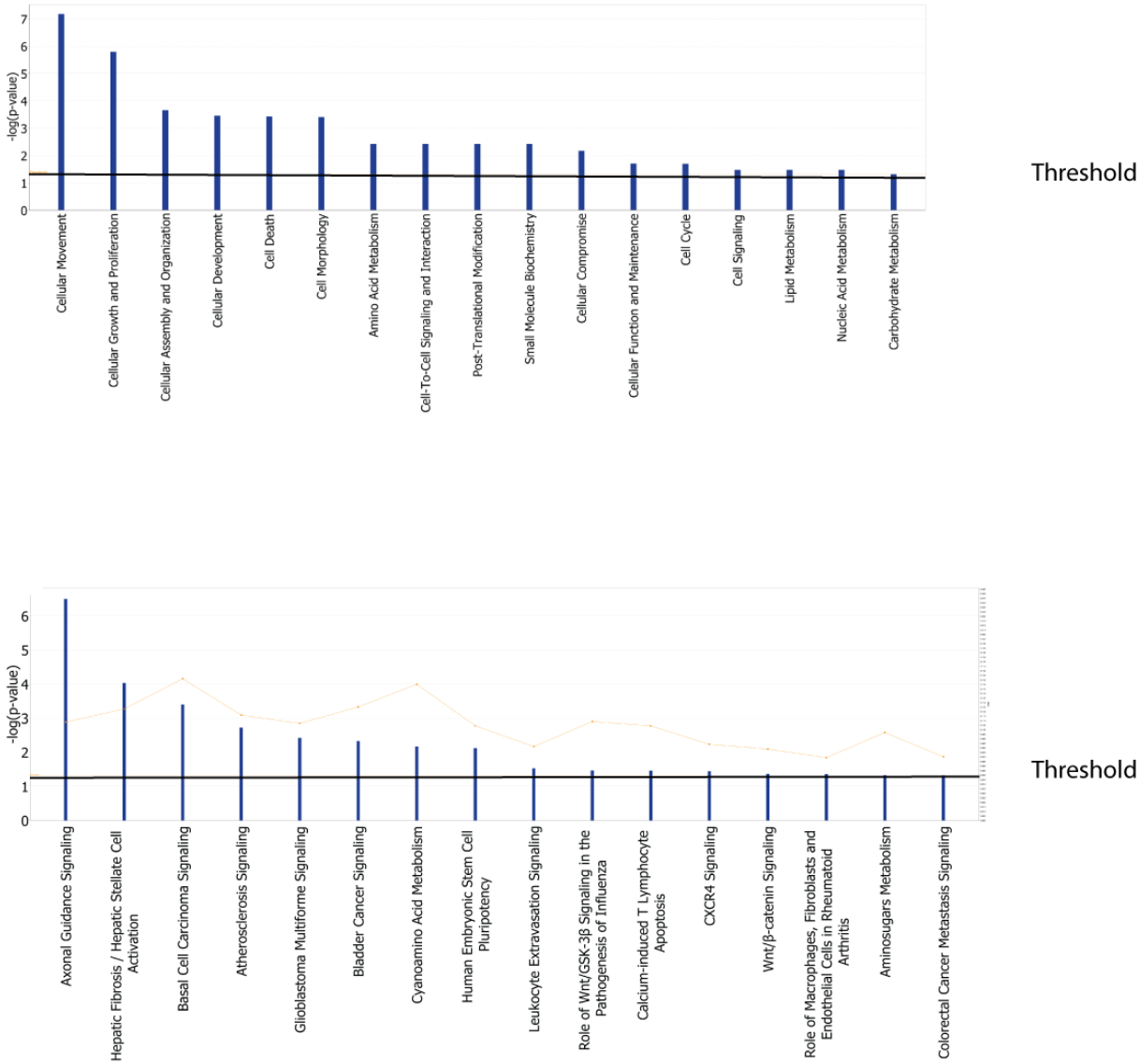


Figure 5.10. IPA analysis of genes differentially expressed between the FP and VLM. **A.** Molecular and cellular functions significantly enriched in the genes differentially expressed in this data set. **B.** Canonical functions significantly enriched by this gene list. The significance of the association between the data set and functions or canonical pathway was measured by a Fischer's exact test. This calculates a p-value determining the probability that the association between the genes in the data set and the function or pathway is explained by chance alone, a p value of <0.05 is considered significant and is marked by the threshold line. For canonical pathway analysis a second method to determine significance is also carried out this is the ratio of the number of differentially expressed transcripts from the data set that map to the pathway divided by the total number of molecules that exist in the canonical pathway (right y axis).

Looking at the list of genes found to be up regulated in the FP relative to the VLM (Table 5.5), Shh is the 20th highest up regulated gene with at 18 fold change between the FP and VLM. Nato3 is the 5th highest. Both of these genes are known to be expressed in the FP, adding confidence to the results of the microarray. The top 60 most up regulated genes are shown in Table 5.5. These provide possible candidate genes for understanding the mechanism involved in midbrain dopaminergic development.

Many of the genes in this table have previously been detected as expressed in the ventral midbrain by *in situ* between E10.5 – E18.5, these are marked with an *. In bold are genes which have been shown to be involved in ventral midbrain development.

Fold Change	**Gene**	**Fold Change**	**Gene**
187.12	ADRA1B* [273]	13.35	LMO7*[274]
83.21	PDLIM3*[275]	12.84	CHRD
77.39	ECM2*[276]	12.18	EFEMP1
67.35	ATP2A3	11.85	VSNL1* [273]
49.70	**FERD3L[244, 249]**	11.73	MAOA [277]
38.74	NXPH1* [276]	11.70	LCAT
31.67	VAV3* [276]	11.38	**HES1[243, 244]**
28.73	PPFIBP2	11.24	FABP3
26.04	MLC1* [276]	10.60	PAP2D
25.71	CRYBA2* [276]	10.39	LOC772356
23.91	FAS	10.38	FOXD3* [278]

21.76	GAL* [273]	10.26	ADCYAP1R1*[279]
21.71	CD247 * [273]	10.18	GDPD5*[276]
21.47	MAP7	9.98	PNAT10
20.89	CAPN8	9.84	CAPN2
20.62	**CALB2[273, 280]**	9.77	AKAP14
20.47	THPO	9.41	LOC415713
19.53	EPB41L3*[273]	9.39	MEOX2
18.27	**SHH[69, 99, 110]**	9.26	LOC768920
18.07	OTOR	8.98	GRIA2*[276]
17.96	ALC	8.70	DLC1
17.15	CKMT2	8.50	SCG2*[277]
17.14	GABBR2[281]	8.50	LOC423138
16.85	FAM38B	8.14	DYDC1
16.44	CELSR1	8.10	**RELN[282, 283]**
16.17	WDR65	8.00	CXorf36
15.44	COL9A1	7.90	DISP1*[104]
14.90	WIF1*[277, 284]	7.79	**LMX1B[131]**
14.30	STK10*[276]	7.78	IGSF11
14.05	TEKT5	7.45	ARHGAP24
13.71	**SLIT2 [285]**		

Table 5.5. Top 60 genes up regulated in the FP relative to the VLM and their fold change. Genes in bold have been described to have a role in midbrain dopaminergic neuron development. * marks genes which have been shown to be expressed in the ventral midbrain**.**

5.3 Discussion

In this Chapter I aimed to determine if Dmrt5 was involved in patterning of the ventral midbrain and infer other possible functions of the Dmrt5 gene during midbrain dopaminergic neuron development.

Comparison of FP, VLM and VLMd transcriptional profiles found that Dmrt5 exogenous expression in the VLM significantly induced a floor plate like transcriptional profile. The precise mechanism by which Dmrt5 induces this profile remains to be elucidated. As endogenous Dmrt5 expression in the FP starts at the time of the microarray analysis, and since this gene is thought to act as a repressor (Xinsheng Nan, unpublished data) Dmrt5 may act to promote a FP like transcriptional profile via repressing more lateral fates. This concurs with findings from investigations using neuronal differentiation of embryonic stems to dopaminergic fate to study the function of Dmrt5 [286]. To further investigate the role of Dmrt5 in the regulation of floor plate transcription, analysis of embryos lacking Dmrt5 as well as microarray analysis of embryos over expressing and null for Dmrt5 at later stages of development would help refine our understanding.

By comparing the analysis of the list of genes showing similar transcriptional profiles in the FP and VLMd, and analysis of all genes significantly differentially expressed in the VLMd, I hoped to identify functions of Dmrt5 which may be important at later stages in development. However the molecular and cellular functions and canonical pathways significantly enriched for in these two data sets are very similar, with the exception of the canonical pathway “Reelin signalling pathway” which is enriched for in the VLMd condition.

Reelin signalling is important for proper migration and positioning of neurons during brain development. Within dopaminergic neuron development, lack of Reelin causes incorrect positioning of the neurons [282, 283]. However, exactly how positioning is affected is not clear and there is contradictory evidence suggesting this pathway may have a role in both radial migration, thought to be involved in the migration of cells from the ventricular zone to the marginal zone [267, 268], and tangential migration which is seen during migration of the developed neurons to target structures [287].

As well as obtaining information on the genes affected by exogenous *Dmrt5* expression, this microarray provides data regarding the transcriptional profile of the floor plate of the developing chick midbrain at HH st 16 (E9.5), the onset of specification . This data set provides a useful source of candidate genes which may be involved the developmental processes occurring in this region at this time. Analysis of this data set identified genes involved in “Cell growth and proliferation” are enriched in this region, as this is a highly proliferative region this is an expected finding. Unexpectedly the FP appears to be enriched for genes involved in “Cell movement”, “Cell adhesion” and “Cell morphology”. In accordance with these biological functions, canonical pathway analysis shows “Axonal guidance” as the most enriched category. These categories were also enriched for in the list of genes differentially expressed upon *Dmrt5* exogenous expression in the VLM.

At the time the tissue was collected for microarray analysis, the FP consists of a pseudo stratified layer of cells which have both apical and basal connections, the nuclei migrate between these two locations, with cell division occurring at the apical side [106, 177]. The first dopaminergic neurons begin to reach the marginal zone at around E10.5 and begin to extend neurites at E11.5 [288]. Therefore the reason so

many genes involved in cell movement, cell adhesion, and axonal guidance are enriched in this region from HH st 16 (E9.5) is not clear.

There is little experimental evidence regarding the process by which the cells transit from the ventricular to intermediate and then marginal zone. It is thought that it occurs via migration of dopamine progenitors along radial glia cells [267, 268]. However this is based on observation of radial glia markers and BrdU labelled TH expressing cells being in close proximity to each other [267], rather than perturbation experiments. The finding that these radial glia cells appear to give rise to dopamine neurons themselves further complicates the situation [106]. Beyond this, there is some evidence that disruption of the attachment of cells to the ventricular zone prevents correct organization of the three layers of the ventral midbrain and affects the proliferation and neurogenesis of midbrain dopaminergic progenitors [177]. Indeed as each layer contains cells in a different stage of development it has long been thought that transition may be linked to developmental stage. In further support of this hypothesis loss of Ngn2 causes cells of the ventral midbrain to remain attached to the apical surface in an undifferentiated state [114, 117].

The gene lists obtained for the FP and VLMd are enriched for genes involved in cell movement, cell adhesion and axon guidance. As such these lists could provide a starting point to better understand how cell transition from one layer to the next. In support of the microarray findings the change in cell morphology seen upon over expression of *Dmrt5* does suggest Dmrt5 has some role in cellular adhesion and movement. Furthermore it would be interesting to try and integrate the findings from Chapter 4 regarding the role of Dmrt5 in neurogenesis and cell proliferation with the transition of cells from the ventricular zone to the marginal zone.

In conclusion the data obtained in this chapter provides a rich source of information on which to base further experiments regarding the role of Dmrt5 in cellular proliferation and differentiation, as well its role in cell movement, adhesion and axonal guidance. The findings of the microarray also provide a description of the genes expressed in the FP at the onset of specification and as such provide a list of candidates which may be important during this process. Furthermore this candidate list of FP genes will allow investigation into the role of cell movement, adhesion and axonal guidance molecules in the FP at this time point.

Chapter 6. Discussion

This thesis set out to investigate the hypothesis that Dmrt5 is involved in midbrain dopaminergic neuron development. This theory was based on 3 pieces of information.

1. Identification of *Dmrt5* from a microarray screen, which compared the expression of the ventral midbrain to more lateral region, at E12.5.
2. The localisation of *Dmrt5* to the region from which dopaminergic neurons arise by *in situ* hybridisation.
3. The role of other Dmrt proteins in development.

This hypothesis was addressed in Chapters 3-5.

In Chapter 3, I characterised the spatial and temporal expression pattern of *Dmrt5* in the chicken embryo during the development of midbrain dopaminergic neurons. I found that *Dmrt5* is expressed within the ventricular zone from HH st16 or E9.5 up until at least HH st34 (~E14.0). The ventricular zone in the midbrain floor plate is the region where proliferating neural progenitors of dopaminergic neurons are found and where expression of genes involved in specification to dopaminergic fate first occurs [106, 115]. Based on this information, regarding the expression of *Dmrt5*, I further refined the hypothesis to investigate whether Dmrt5 is involved in maintaining the progenitor population, neurogenesis or specification of these cells to dopaminergic fate.

In Chapter 4, I used an inducible *Dmrt5* epistem cell line to over express *Dmrt5* during differentiation of these cells to dopaminergic fate. For *in vivo* studies I used *in ovo* electroporation to exogenously express *Dmrt5*. These studies identified that

exogenous *Dmrt5* leads to a decrease in the number of neurons, as detected by antibody staining for Tuj1, a decrease in the expression of proneural gene, *Ngn2* as detected by real-time PCR, and a decrease in cell proliferation with cells being held in a G1 retarded stage. The up regulation of *Hes1* in the mouse telencephalon also results in these effects [251]. Furthermore, *Hes1* was found to be up regulated by *Dmrt5 in vitro* in epistem cell-derived neural progenitors and is ectopically expressed *in vivo* upon exogenous expression of *Dmrt5*. Conversely, the Hes1 repressor *Nato3* was down regulated in mouse epistem cell model upon over expression of *Dmrt5*.

Previous literature has described a direct regulation of Hes1 homolog, *Ref-1*, by Dmrt homolog Mab-3 in C.elegans [242]. Moreover, expression of Drosophila Dmrt homolog, dsx, in C.elegans had the same effect, suggesting that this direct regulation was conserved [192]. To investigate if mouse Dmrt5 directly regulates mouse *Hes1*, I searched for Dmrt5 consensus binding sites in the region upstream of *Hes1* transcriptional start site. Dmrt5 binding sites were found and initial ChIP experiments suggest Dmrt5 binds to these sites with greater affinity than non-specific antibodies. These experiments indicate that Dmrt5 can regulate *Hes1* directly. This work was described in Chapter 4.

In Chapter 5 I studied the genome-wide effect of over expressing *Dmrt5* ectopically in the ventral lateral midbrain of HH st 10 chicks for 24 hours. As *Dmrt5* is expressed in the ventricular zone and is involved in neurogenesis and regulation of the cell cycle I wanted to investigate if it was involved in patterning of the ventral midbrain. As described in the introduction, several other genes that regulate expression of Ngn2, a key mediator in midbrain dopaminergic neurogenesis, are also involved in repressing lateral fates of the Red Nucleus (RN) and Ocular Motor (OCM) neurons and promoting the ventral midbrain fate, which is permissive to dopaminergic neuron

development. Examples of this include Lmx1A [103], FoxA2 in combination with Lmx1A [118, 123, 124] and Otx2 [122, 139].

By comparing the transcriptional profile of the floor plate (FP) and that of VLM ectopically expressing *Dmrt5,* I found that *Dmrt5* ectopic expression did induce a profile similar to the floor plate. Therefore, *Dmrt5* is involved in patterning of the ventral midbrain. Furthermore, IPA analysis revealed that FP-enriched and Dmrt5 induced genes are involved in cell growth and proliferation, cell movement, cellular attachment and adhesion were affected in both conditions. In accordance with these findings, ectopic expression of *Dmrt5* in the ventral midbrain cells resulted in an unusual morphology where cells were not attached to the basal or apical layers.

In summary, Dmrt5 regulates expression of *Hes1* and neurogenesis, affects cell proliferation, cell movement, adhesion and migration. These findings strongly implicate a role for Dmrt5 in development. Further research, including loss of function studies, will now be able to build on from this work to determine the precise role of Dmrt5 in these processes.

In the following sections I will discuss aspects of the thesis in more detail and discuss the findings in the context of the current literature.

6.1 The role of Hes1 in midbrain dopaminergic development

Hes1, a basic Helix-Loop-Helix (bHLH) gene, inhibits expression of proneural genes, is required for maintenance of neural progenitors and regulates the timing of their differentiation into neurons [246-248]. Thus in the nervous system, Hes genes are critical for generating the correct numbers and full diversity of neurons and glial cells

[248, 289]. This thesis provides evidence that Dmrt5 directly regulates *Hes1* and has a role in neurogenesis of the ventral midbrain.

The role of Hes1 in ventral midbrain development has only recently been described in three papers [243, 244, 290]. All papers find perturbation of Hes1 affect neurogenesis and cell proliferation in the ventral midbrain [243, 244, 290]. Ono et al showed that Nato3 is required for cell cycle progression and induction of proneural genes, Ngn2 and Mash1, in the ventral midbrain via repression of *Hes1* [244]. This paper states that Hes1 expression is no longer detected at E10.5 and that this loss of Hes1 is mediated by direct repression by Nato3 [244]. Lahti et al, demonstrate that FGF signalling is needed for Hes1 expression in the ventral midbrain at E10.5 and E11.5. Loss of FGFR1/2 reduces Hes1 expression and results in premature neurogenesis and an increase in exit from the cell cycle [243]. In the third study, Hes1 null mice were analysed. These mice still develop some dopaminergic neurons expressing both Pitx3 and TH. However neurogenesis is precocious and depletion of the neural progenitor pool leads to a reduced number of dopaminergic neurons later in development [290]. As Hes1 has a role in formation of the IsO which is essential for correct formation of ventral midbrain [291], it is possible that some of these defects are due to loss of this structure. Although the details regarding the role of Hes1 in the ventral midbrain require further clarification the findings of this thesis are consistent with the current literature with up regulation of Hes1 seen upon exogenous expression of *Dmrt5* affecting neurogenesis and cell proliferation. In the future conditional knock out of Hes1 in the ventral midbrain after formation of IsO will better define the role of Hes1 in ventral midbrain neurogenesis.

The role of Nato3 and Hes1 in neurogenesis is postulated by Ono et al to be distinct from the previously described neurogenic network involving Lmx1A. This proposal is

based on the observation that in the absence of Nato3, midbrain dopaminergic neurons are correctly specified, expressing Lmx1A/B, Pitx3, Nurr1 and TH, with only the number of neurons being affected [244]. Dmrt5 affects patterning and neurogenesis, further investigation will be required to determine if these two roles are distinct or interlinked.

6.2 Dmrt5 as a general regulator of neurogenesis

While there is little information regarding the role of Dmrt genes outside of sexual differentiation and development, what information there is indicates that Dmrt genes may have a conserved role in regulation of neurogenesis. Thus far, three publications have implicated Dmrt and Dmrt homologs in neurogenesis in Xenopus, Drosophila and C.elegans. This thesis describes the role of Dmrt5 in neurogenesis, in both the chicken and mouse, adding further support for this hypothesis.

In Xenopus, Dmrt4 positively regulates genes involved in neuronal differentiation of the olfactory epithelium Xebf2, neurogenin and NCAM [225]. In C.elegans Dmrt homolog, Mab-3, represses Ref-1, a Hes1 homolog [292], to enable expression of proneural gene Lin32 (a Mash homolog) and V ray neurogenesis [242]. While in Drosophila, Dmrt homolog, Dsx, is known to regulate abdominal neuroblast differentiation [250].

The mechanism by which neuronal markers are up regulated by Dmrt4 in the olfactory epithelium of Xenopus is not known. However, it may be possible that as postulated by Ross et al, regulation of neurogenesis by Dmrt proteins is mediated by regulation of Hes genes [242]. In support of this, expression of the male Drosophila DSXm, in C.elegans, rescues the Mab-3 mutant phenotype by inhibiting the ectopic

Ref-1 expression seen in Mab-3 mutants [192]. This suggests that these 2 proteins can both regulate the Hes1 homolog, Ref-1. As we learn more about the role of Dmrt genes outside of sexual development, studies will reveal if regulation of bHLH proteins and in particular Hes family members by DM domain transcription factors is a conserved mechanism.

It is interesting to note that while *Dmrt5* ectopic expression results in an increase in *Hes1* expression and a subsequent reduction in neurogenesis. In C. elegans and Xenopus, loss of Mab-3 and Dmrt4 respectively inhibit neurogenesis, while ectopic expression of these genes promotes neurogenesis. These findings indicate that in these contexts Dmrt genes promote neurogenesis. Similarly in Drosophila in the absence of Dsx sex specific neuroblast fail to undergo divisions in male or female larval nervous systems suggesting that Dsx is required for generation of these neurons [250]. It will be interesting to investigate the effect of Dmrt5 loss-of-function on neurogenesis and why in the context of the midbrain Dmrt5 seems to have the opposite effect on neurogenesis.

6.3 Is Dmrt5 transcription factor an activator or a repressor?

Initial ChIP experiments demonstrate that Dmrt5 binds to the *Hes1* promoter and that upon *Dmrt5* exogenous expression *Hes1* up regulation or ectopic expression is observed. These results suggest that Dmrt5 is directly activating expression of *Hes1*. This is unexpected for two reasons. Firstly in C.elegans, although Mab-3 directly regulates transcription of *Ref-1*, this is a direct repression of Hes1 homolog, *Ref-1,* rather than activation. Secondly, Dmrt5 is thought to be a transcriptional repressor as fusion of mouse Dmrt5 to VP16 activated transcription of targets in transfected

Hek293T cells [208]. Indeed, VP16 fusions to all 6 mouse Dmrt genes showed the same effect as Dmrt5, suggesting that Dmrt genes are repressors of transcription [208].

In support of our finding that Dmrt5 is acting as an activator of *Hes1*, there is evidence that Dmrt genes can act as both activators and inhibitors, depending on the tissue in which Dmrt genes are expressed [206, 242]. Furthermore, although Dmrt homolog in C.elegans, Mab-3, was found to repress transcription of Hes1 homolog, *Ref-1*. This repression was in part, hypothesised to be a result of antagonising activation by some unknown protein which binds to an overlapping site [242].

It is postulated that the binding of Dmrt proteins to the minor groove of DNA enables simultaneous binding of major groove factors to the same region of the enhancer element [206]. One example where this has been described is in Drosophila genes Dsx-f and Dsx-m, which can activate or repress expression of their transcriptional target Yp1 respectively [206]. Dsx-m has a longer C terminal domain which is proposed to occlude the binding site of a bZIP activator to the major groove. In support of this hypothesis, disruption of Mab-3 binding sites in the promoter of *Ref-1* prevents the activation of this gene, even though Mab-3 inhibits expression of *Ref-1*. This finding suggests that the activator binds in close proximity to the Mab-3 site, which may in turn indicate that Mab-3 represses *Ref-1* by physically interfering with binding or function of activators bound to nearby sites [242]. The structure of Dmrt proteins may be particularly suited for interaction with transcription factors that bind overlapping sites of DNA, as Dmrt proteins bind to the minor groove [204] which could allow close apposition with major groove binding transcription factors.

In further support of the role of Dmrt5 in gene activation the results of the microarray show that 1229 genes are up regulated by ectopic expression of *Dmrt5*, while only 229 are down regulated. Although not all of these changes will be the result of direct transcriptional targeting, one may have expected if Dmrt5 is solely a transcriptional repressor, that the number of down regulated genes would have been larger.

In conclusion, it appears that Dsx can act as a repressor or an activator [206], and in the context of my thesis Dmrt5 appears to act as an activator.

6.4 Dmrt5, an example of genes involved in midbrain dopaminergic neuron development.

Like several other genes involved in ventral midbrain development, Dmrt5 seems to affect several processes, cell proliferation, cell patterning, and neurogenesis. The gene network in Figure 1.4 of Chapter 1 depicts a model of the regulatory network involved in development of midbrain dopaminergic neurons. Many genes involved in regulation of neurogenesis also function to inhibit the expression of more lateral fates, thus promoting a ventral phenotype. For example Lmx1A has been found to indirectly activate Ngn2, thus regulating neurogenesis [121]. Lmx1A also prevents expression of Red Nucleus (RN) fate specification genes [123]. Expressions of the genes involved in specification to RN fate inhibit dopaminergic fate and thus the repression of RN specification genes is crucial for correct neurogenesis of dopaminergic neurons. In particular Ngn1 expressed in the RN fate lineage represses Pitx3, a gene required for correct dopaminergic fate [123, 132]. FoxA2 in combination with Lmx1A is required for this inhibition of RN fate [123]. FoxA2 therefore is another example of this mechanism. FoxA2 is involved in neurogenesis, via regulating Ngn2 expression, but also prevents expression of more lateral fates by

repressing Ngn1 [123], Nkx2.2 [124] and Helt [124] a gene expressed in neurons of GABAergic fate. A third example of this two pronged regulation of midbrain dopaminergic neuron development is Otx2 which regulates expression of Ngn2 and thus neurogenesis but also inhibits expression of Nkx6.1 which is expressed in more lateral regions [122, 139].

Dmrt5 regulates expression of Ngn2 and neurogenesis, and microarray studies have also found that it represses expression of lateral fates, while significantly promoting expression of a ventral phenotype. Therefore Dmrt5 is another gene which fits this model and so is an example of the mechanisms by which midbrain dopaminergic specification genes give rise to dopaminergic neurons via regulation of Ngn2 and repression of more lateral fates.

6.5 What initiates Dmrt5 Expression?

This thesis investigates the role of Dmrt5 in ventral midbrain development and genes regulated by Dmrt5. However what triggers expression of Dmrt5 is not known. The expression of *Dmrt5* starts in the anterior of the midbrain with posterior expression occurring later, this anterior to posterior pattern of expression mirrors the trend seen in the birth of *TH* expressing cells [293]. Genes with a similar spatial expression pattern that are expressed prior to Dmrt5, coupled with analysis of the region up stream of the Dmrt5 transcriptional start site for binding site of such genes may help to identify what activates expression of *Dmrt5*. Furthermore, investigation of the expression of Dmrt5 in mutant embryos such as FoxA2/1, Shh, Otx2, Lmx1A and Wnt null mice could help us to understand which genes/signalling pathways are involved in regulating *Dmrt5*.

6.6 Future directions

Less neuron are seen when *Dmrt5* is exogenously expressed both in the chicken midbrain and in epistem cell-derived neural cultures differentiated to dopaminergic fate as measured by antibody staining and real-time PCR, respectively. In agreement with this reduction in neurons, loss of proneural markers, such as Ngn2 and Mash1, in combination with the reduction in cell proliferation is also seen. However this phenotype could also be caused by cell death. The findings from the microarray do not show an increase in the number of cell death genes in *Dmrt5* over expressing ventral midbrain tissue compared to unelectroporated or mock electroporated controls. Furthermore, while a reduction in neuronal markers and markers involved in specification to dopaminergic fate are seen upon *Dmrt5* exogenous expression, the effect of *Dmrt5* expression on TH has not been investigated. One would expect that there would be a reduction in the number of TH positive neurons, however this should be analysed.

As discussed earlier in this Chapter there are some discrepancies regarding the temporal expression pattern of Hes1 during midbrain development. Ono et al show expression is below detection at E10.5, while Lahti et al show expression of Hes1 at E11.5. To better understand the regulation of *Hes1* by Dmrt5, the expression pattern of both of these genes should be investigated further by double *in situ* or antibody staining. Furthermore, while I have mapped the expression pattern of *Dmrt5* during the time window of specification and differentiation of the ventral midbrain to dopaminergic neurons further investigation of *Dmrt5* expression both earlier and later in development should be carried out to identify any other roles of this gene.

To complement these gain of function studies which provide insight into the possible functions of Dmrt5, loss of function studies are also essential. A Dmrt5 heterozygous mouse has recently been generated in the lab, and is currently being studied. Interestingly in the Dmrt5 knock out cell line, cells differentiated to dopaminergic fate also show an up regulation of *Hes1* (Nicole Gennet). This suggests that there is an added complexity in the regulation of *Hes1* by Dmrt5 during midbrain dopaminergic neurogenesis. The neurogenic effect of loss of Dmrt5 has not yet been investigated further.

On a conceptual level inhibiting neurogenesis in cells just as this process is being initiated is counterintuitive. However, as is seen in the Hes1 mutant this is an essential process required to ensure that the progenitors continue to divide and thus give rise to the correct number of neurons. Based on the findings of this thesis I would propose that Dmrt5 functions to maintain neural progenitors and prevents their terminal differentiation via its regulation of *Hes1*. Further studies are required to confirm this.

The microarray data presented in this thesis provides a rich source of information for future studies. The biggest challenge when analysing this is to decide what questions to ask in order to find meaningful results which will help further our understanding of Dmrt5. One important study would be to identify genes directly regulated by Dmrt5. To achieve this, the genes up and down regulated by *Dmrt5,* as found by microarray analysis, should be analysed to find those which contain Dmrt5 consensus binding sites. This candidate list of Dmrt5 target genes can be further refined by identifying which of these are also found in microarray data from the over expression and knock down of *Dmrt5* in dopaminergic neurons derived from embryonic stem cells *in vitro*. Whilst the differentiation of embryonic stem cells to

dopaminergic fate may lack some of the developmental patterning and possibly cofactors required for correct Dmrt5 function, comparison of this data to the *in vivo* over expression of *Dmrt5* may help to refine the genes which are directly regulated by Dmrt5. Furthermore, ChIP Seq experiments could be carried out to identify more direct targets of Dmrt5.

With regard to the FP data from the chicken embryo collected at HH st 16, comparison of this to microarray data obtained in the lab from the FP of mouse at E9.5 can be used to identify both similarities and differences in the transcriptional profile of this tissue across species. Genes conserved in both species and so far unknown in this region may provide good candidates for future investigations into genes involved in midbrain dopaminergic neuron development.

While this thesis concentrated on identifying a role of Dmrt5 in midbrain development, the microarray data provide useful information regarding the function of Dmrt5 in other tissues. This is because it provides a list of genes which are regulated, directly or indirectly, by Dmrt5. For example in Chapter 3, I identified that *Dmrt5* is expressed in the optic placodes and the lens of the developing chicken embryo. From the microarray analysis I found *Dmrt5* ectopic expression leads to the up regulation of many genes involved in axon guidance. One of these genes Sema3A is expressed in the lens and is required for correct eye development [294]. Sem3A signalling from the lens segregates periocular neural crest into 2 lineages. A Npn-1 negative lineage which is essential for periocular neural crest migration and formation of the cornea and a npn1 positive lineage which is needed for formation of anterior uvea and periocular tissues [239]. Since *Dmrt5* is expressed in the lens and up regulates Sema3A it may be involved in this process. My data set could be of use

for those interested in the formation of other Dmrt5 expressing tissues such as the eye, telencephalon, optic stalk nasal placodes and pituitary.

6.7 The significance of the findings presented in this thesis.

Although a collection of genes are known to be involved in neurogenesis of the ventral midbrain, our understanding of the mechanisms causing neurogenesis in this region is still incomplete. *Dmrt5* is expressed in the region from which midbrain dopaminergic neurons arise within the ventral midbrain, in the same region as *Hes1*. Here we show Dmrt5 binds to the promoter region of *Hes1* and can ectopically induce *Hes1* expression. Furthermore *Dmrt5* affects cell proliferation and neurogenesis, both of these processes are also affected by Hes1, implying the effects observed upon *Dmrt5* over expression may in part be mediated via regulation of *Hes1*. *Dmrt5* also causes the down regulation of *Nato3,* a gene known to repress Hes1 expression and induce neurogenesis. As such Dmrt5 is a new member of the neurogenic pathway of the ventral midbrain. Furthermore, microarray screens followed by candidate validation by real-time PCR showed that *Dmrt5* promotes a ventral transcriptional profile. Therefore Dmrt5 is a gene involved in three fundamental aspects of ventral midbrain development: patterning, neurogenesis and cell cycle of ventral midbrain neurons. The information in this thesis improves our understanding of the genes involved in dopaminergic neuron development. This information can be employed to improve current *in vitro* midbrain dopaminergic differentiation methods so that phenotypically correct dopaminergic neurons can be created *in vitro* efficiently. These can be used for disease modelling, drug screens and even transplantation.

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