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DMRT5 Together with DMRT3 Directly Controls Hippocampus Development and Neocortical Area Map Formation

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Abstract

Mice that are constitutively null for the zinc finger doublesex and mab-3 related (Dmrt) gene, Dmrt5/Dmrt2, show a variety of patterning abnormalities in the cerebral cortex, including the loss of the cortical hem, a powerful cortical signaling center. In conditional Dmrt5 gain of function and loss of function mouse models, we generated bidirectional changes in the neocortical area map without affecting the hem. Analysis indicated that DMRT5, independent of the hem, directs the rostral-to-caudal pattern of the neocortical area map. Thus, DMRT5 joins a small number of transcription factors shown to control directly area size and position in the neocortex. Dmrt5 deletion after hem formation also reduced hippocampal size and shifted the position of the neocortical/paleocortical boundary. Dmrt3, like Dmrt5, is expressed in a gradient across the cortical primordium. Mice lacking Dmrt3 show cortical patterning defects akin to but milder than those in Dmrt5 mutants, perhaps in part because Dmrt5 expression increases in the absence of Dmrt3. DMRT5 upregulates Dmrt3 expression and negatively regulates its own expression, which may stabilize the level of DMRT5. Together, our findings indicate that finely tuned levels of DMRT5, together with DMRT3, regulate patterning of the cerebral cortex.

Key words: cortical hem, hippocampus, neocortex, neocortical area map, transcription factor

Introduction

Cerebral cortex is divided into 6-layered neocortex, paleocortex or olfactory cortex, and archicortex, comprising the hippocam-pus and perihippocampal fields. The hippocampus is key to memory, and distinct areas within the neocortex mediate higher functions such as perception, attention, and behavioral planning (Nauta and Feirtag 1986). Genetic defects influencing early cortical development underlie many human neuropsychiatric and neurological disorders (Gaitanis and Walsh...
2004; Hu et al. 2014), and, particularly germane to the present study, human DMRTA2/DMRT5 has recently been associated with microcephaly and region-specific neocortical pachygyria (Urquhart et al. 2016).

Embryonic patterning of the cortex begins with the diffusion of signaling molecules from signaling centers (Crossley et al. 2001; Ohkubo et al. 2002; Mangale et al. 2008; Toyoda et al. 2010). Fibroblast growth factors (FGFs) disperse from the rostral telencephalic patterning center (RTPC) (Crossley and Martin 1995; Bachler and Neubuser 2001; Storm et al. 2006; Cholfin and Rubenstein 2007), and WNT and bone morphogenetic proteins (BMPs) from the dorsomedial roof plate and cortical hem (Furuta et al. 1997; Grove et al. 1998). FGFs from the RTPC specify areas of rostral neocortex and establish the rostrocaudal axis of the entire neocortex, whereas the hem is required for development of the hippocampus and caudo medial neocortex and helps position the boundary between neocortex and paleocortex (Lee et al. 2000; Fukuchi Shimogori and Grove 2001; Garel et al. 2003; Yoshida et al. 2006; Cholfin and Rubenstein 2007, 2008; Mangale et al. 2008; Toyoda et al. 2010; Caronia-Brown et al. 2014).

WNT, BMP, and FGF signaling interactively regulate in cortical progenitor cells the expression gradients of several transcription factor genes implicated in cortical patterning. These include the home o box genes Lhx2, Pax6, and Emx2, and the zinc finger double sex and mab-3 related (Dmrt) gene, Dmrt5/Dmrt2a (Monuki et al. 2001; Muzio et al. 2005; Cholfin and Rubenstein 2008; Konno et al. 2012; Saulnier et al. 2013; Caronia-Brown et al. 2014). Lhx2 has several functions in cortical patterning, first as a selector gene specifying cortical identity (Mangale et al. 2008) and subsequently determining the division between neocortex and paleocortex (Lee et al. 2000; Fukuchi Shimogori and Grove 2001; Garel et al. 2003; Yoshida et al. 2006; Cholfin and Rubenstein 2007, 2008; Mangale et al. 2008; Toyoda et al. 2010; Caronia-Brown et al. 2014). Lhx2 is required for normal development of primary somatosensory cortex (S1) (Shetty et al. 2013). Pax6 is expressed in a rostro lateral high to caudo medial low gradient in the cortical primordium (CP) and promotes development of rostral areas at the expense of caudal areas (Bishop 2000; Muzio et al. 2002), possibly by differentially regulating cell division along the rostral-to-caudal axis (Gotz et al. 1998; Manuel et al. 2007; Asami et al. 2011; Mi et al. 2013). Additionally, Pax6 contributes to the development of a normally sized S1 (Zembrzycki et al. 2013). Emx2 is expressed in an opposite caudo medial high to rostro lateral low gradient and, consistent with this gradient, regulates the size of the hippocampus and caudo medial neo-cortex (Yoshida et al. 1997; Mallamaci et al. 2000; Tole et al. 2000; Hamasaki et al. 2004; Muzio et al. 2005). To date, whether Dmrt5, expressed in the same gradient as Emx2, directly plays a similar role is unclear.

In mice constitutively lacking Dmrt5, the hippocampus is missing, caudomedial neocortex is extremely reduced, and paleo-cortex expands dorsally. Additionally, in Dmrt5−/− mice, the cortical hem is virtually gone, suggesting that Dmrt5 controls cortical patterning indirectly by promoting cortical hem formation (Konno et al. 2012; Saulnier et al. 2013). Yet, Dmrt5 expression is upregulated by WNT signaling and downregulated by FGF8 (Konno et al. 2012; Saulnier et al. 2013; Caronia-Brown et al. 2014), implying that DMRT5 may also have a second direct role in cortical patterning. It remains unknown therefore, whether DMRT5 itself influences the neocortical area map and whether different levels of DMRT5, induced experimentally, would affect the area map in a “dose-dependent” manner, as is true for EMX2 (Hamasaki et al. 2004; Zembrzycki et al. 2015).

To gain a better understanding of DMRT5’s mode of action in cortical development, we generated mice with floxed Dmrt5 and mated them with mice carrying different Cre alleles. In all cases, the stage of Dmrt5 deletion followed the initial formation of the cortical hem, just after mid-gestation. In both NestinCre and Emx1-Cre cKO mice, Dmrt5 was deleted in the CP. In Nestin Cre cKO animals, however, DMRT5 remained in the cortical hem, whereas it was diminished in Emx1-Cre cKO mice. To delete Dmrt5 from the hem only, Dmrt5 floxed mice were mated with Wnt3a-Cre mice. Comparing
the phenotypes of the progeny revealed that at this developmental stage, hippocampal development and neocortical patterning requires DMRT5 in the CP but not in the hem.

For conditional gain of function of Dmrt5, we generated a transgenic line that, when crossed with Emx1-Cre mice, generated progeny with excess Dmrt5 expression in the CP. Altering the distribution and levels of DMRT5 led us to conclude that 1) DMRT5 controls patterning of the caudal cortex independent of its influence on the hem, 2) size and position of neocortical areas is sensitive to different levels of DMRT5, and 3) DMRT5 negatively controls Dmrt5 expression suggesting negative autoregulation.

Finally, we examined the genetic association of Dmrt5 with Dmrt3, related genes that have comparable expression gradients in cortical progenitor cells. Dmrt3 null mice show cortical defects, but these are much milder than those Dmrt5 null. We tested interactions between the Dmrt genes by assessing their expression in Dmrt3 and Dmrt5 mutant lines. Expression of Dmrt5 increases in the absence of Dmrt3, possibly explaining the more modest cortical phenotype in the Dmrt3 null and demonstrating that Dmrt5 expression is negatively controlled not only by DMRT5 but also by the related transcription factor DMRT3.

Materials and Methods

Mouse Lines and Genotyping

All animals were maintained in a C57Bl/6 background except for CD1 mice used for in utero electroporation studies. Midday of the day of vaginal plug discovery was termed embryonic day 0.5 (E0.5), and the first 24 h after birth was P0. Animal care was in accordance with institutional guidelines, and the policies of the US National Institutes of Health.

The Dmrt5 conditional knockout allele (Supplementary Fig. 1A) was generated using standard recombinering methods. A bacterial artificial chromosome containing the Dmrt5 gene as well as linearized plasmid DNA containing short Dmrt5 flanking sequences were electroporated into Escherichia coli induced to express homologous recombination machinery. Homologous recombination generated a plasmid containing 11 kb of the mouse Dmrt5 locus encompassing exons 1–3. Next, loxP sites were inserted in this plasmid by homologous recombination into the first and second introns of Dmrt5 together with a neomycin resistance gene (NeoR). This plasmid was linearized and electroporated into mouse ES cells to generate the Dmrt5 conditional mutant allele. ES cells containing the Dmrt5 conditional knockout allele were provided to the University of Minnesota Mouse Genetics Laboratory for the generation of chimeric mice. Highly chimeric mice were bred to wild-type females. To remove the NeoR gene from the Dmrt5 locus, mice containing the Dmrt5 conditional knockout allele were bred to mice containing a ubiquitously expressed FlpE recombinase enzyme. This generated mice carrying the final Dmrt5floxed(fl) allele (Supplementary Fig. 1A). Dmrt5fl/fl mice were crossed to Emx1-IRES-Cre (Gorski et al., 2002), NestinCre (Tronche et al. 1999), or Wnt3a-IRES-Cre mice (Yoshida et al. 2006). To distinguish between the wild-type and Dmrt5 floxed alleles, mice were genotyped by PCR using genomic DNA from the tail and primers: Fwd 5′-TCTCTGTAACTCAGTCCCTTCAGG-3′; Rev 5′-GTAC TTCTCCGGCGCCTCAAC-3′ (Supplementary Fig. 1A, primers a, b “Dmrt5 Lox”). To detect the Cre-excised allele, mice were genotyped by PCR using the following primers on genomic DNA from dorsal telencephalon: Fwd 5′-TCTCTGAATCAGTCCC TTCCAGG-3′; Rev 5′-AGGAAAGGAATCTTGCTGAAACG-3′ (Supplementary Fig. 1A, primers a, c “Dmrt5 Δ2”).

To generate Dmrt3 null mutants, we constructed a targeting vector in which exon 1 of Dmrt3 was replaced by NeoR flanked by loxP sites. After isolation of homologous recombinants in ES cells, the
clones were used to generate chimeric mice that were mated to WT mice to generate heterozygous animals. The NeoR cassette was removed by crossing the Dmrt3neo/+ heterozygous mice with beta-actin Cre mice. For genotyping, primers used were: Fwd (WT) 5’-ATGAACGGCTACGGCTCCCCCTAC-3’; Fwd (KO) 5’-GGAAAGCGGTCTAGGGCTCAGTGC-3’; Rev 5’-CCCGGGGAAGACCTCTGACGATAG-3’ (Supplementary Fig. 1B).

For Dmrt5 gain of function, a Dmrt5 conditional transgenic mouse model was generated using the Gateway-compatible ROSA26 locus targeting vector as previously described (Nyabi et al. 2009). The DNA coding sequence corresponding to Dmrt5 was isolated from pFLCI EST clone: B130012I23. The Dmrt5 transgene was targeted to the ROSA26 locus, downstream of a floxed cassette containing a neomycin resistance marker and a transcriptional stop element. To tag transgene-expressing cells with eGFP, an IRES-eGFP element was inserted at the C-terminus of the transgene (Nyabi et al. 2009) (Supplementary Fig. 1C). To distinguish between the wild-type and Dmrt5 trans-gene allele, mice were genotyped by PCR using primers: Fwd Rosa26 5’-AAACTGGCCCTTGCCATTGG-3’; Fwd eGFP 5’-AACGAGAAGCGCGATCACAT-3’; Rev Rosa26 5’-GTCTTTAATCTACGTGATGG-3’ (Supplementary Fig. 1C, primers d, f, g “Dmrt5Tg”).

To distinguish between the floxed targeted allele (Dmrt5Tg) and the Cre-excised allele, the following primers were used: Fwd Rosa26 5’-AAACTGGCCCTTGCCATTGG-3’; Rev Neo5’-CTCGTGTCATGTCAGTTCATTCA-3’; Rev Dmrt5 5’-TGCCTGCGCAAGGCCACC TGA-3’ (Supplementary Fig. 1C, primers d, e, h “Dmrt5ΔNeo”). In both cases, the presence of Cre recombinase was assessed with primers: Fwd 5’-GTTCGCAAGAACCTGATGGA-3’; Rev 5’-CCACCCTACCTGAGAT-3’. Crossing Dmrt5 conditional transgenics (termed here Dmrt5Tg mice) with Emx1-Cre mice induced in the dorsal telencephalon both eGFP fluorescence and excess DMRT5 protein (Supplementary Fig. 1D,E).

Generation of DMRT5 Antibodies

Polyclonal antibodies for mouse DMRT5 were generated by immunizing rabbits with the 2 following peptides: GRPDSPQPPPGBPSPGPRC (aa 200-223) and CKEPGYGGGLYGPMVNGTPEKQ (aa 511-531) (PSL Peptide Specialty Laboratories, GmbH).

Histology and Immunofluorescence

Standard hematoxylin and eosin (H&E) staining was performed on 6–8 µm sections of embryos or brains fixed overnight in 4%paraformaldehyde/PBS, dehydrated and paraffin-embedded.

For immunofluorescence, embryos were fixed overnight in 4%paraformaldehyde/PBS, infused in 30% sucrose/PBS overnight, frozen in gelatin (7.5% gelatin, 15% sucrose/PBS) and sectioned in 20 µm cryostat sections. Antigen retrieval was performed by boiling the sections in Target Retrieval Solution Citrate pH 6.0 (DAKO). Sections were then permeabilized in 0.3% Triton X-100 and blocked in 10% goat serum. Primary antibodies used were: DMRT5 (rabbit, 1/2000, gift from Dr Meng Li, Cardiff), CTIP2 (rat, 1/250, abcam ab18465), PAX6 (mouse 1/100, DSHB), β-TUBULIN (mouse, 1/100, Covalent). Secondary antibodies used were: Alexa Fluor 488 goat anti-rabbit (1/400, A-11008, Invitrogen), Alexa Fluor 594 goat anti-mouse (1/400 A11005, Invitrogen) and Alexa Fluor 594 anti-rat (1/400, A11007, Invitrogen). Sections were counterstained with DAPI. Wide-field images were acquired with a Axio Observer Z1 microscope.

In Situ Hybridization

In situ hybridization (ISH) on sections was performed using anti-sense digoxigeninlabeled riboprobes as described (Saulnier et al. 2013). Dmrt5 expression in Emx1 cKO embryos and in Dmrt3 KO embryos were analysed using an antisense probe corresponding to the 3’ half of exon three
synthesized by linearizing EST AI592924 (Genbank) with EcoRI and transcribing it with T3 as previously described (Saulnier et al. 2013). Endogenous Dmrt5 expression in Dmrt5Tg mutants was evaluated using an antisense probe corresponding to a portion of the 3′ UTR generated by linearizing the same EST with BamHI and transcribing it with T3. The other antisense probes were generated from the following previously described cDNA clones: Lmo3, Lmo4 (Bulchand et al. 2001), Cdh6 (Bishop 2000), and tdTomato (Genove et al. 2005). For whole-mount ISH, brains stored in methanol 100% were rehydrated and treated with a 6% hydrogen peroxide solution for 60 min. Brains were then incubated with 20 µg/ml proteinase K for 45 min (E18.5) or 55 min (P7) and post-fixed 20 min in 4% paraformaldehyde/0.2%glutaraldehyde. Brains were incubated in hybridization solution (50% formamide, 5X SSC, 10% (E18.5) or 20% (P7) SDS, 500 µg/ml tRNA, 200 µg/ml acetylated BSA, 50 µg/ml heparin) for at least 1h at 70°C, then hybridized to digoxigenin labeled riboprobes (1–2 µg/mL) in hybridization solution overnight at 70 °C. The next day, brains were washed in solution X (50% formamide, 2X SSC, 1% SDS) 4 × 45 min at 70 °C and equilibrated in MABT (0.1 M maleic acid, 150 mM NaCl, 1% Tween-20, pH 7.5) 3 × 10 min. Brains were then blocked 2 h in blocking solution (10% lamb serum, 2% blocking reagent (Roche) in MABT) and incubated with anti-digoxigenin antibody (1/4000, Roche) in blocking solution overnight at 4 °C. The next day, brains were washed at least 5 × 1 h and then overnight in MABT. Finally, brains were incubated 3 × 10 min in NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM MgCl2,1%Tween-20, 2 mM Levamisole) and detection of the anti-digoxigenin antibody was performed by NBT/BCIP solution at room temperature. Images were acquired with an Olympus SZX16 stereomicroscope and an Olympus XC50 camera, using the Imaging software Cell®.

Quantification of the dorsal surface area of the cortical hemi-sphere of E12.5, E18.5, P7, and adult animals was obtained by taking measurements from images of whole brains. Measurement of the surface areas of primary motor, sensorimotor, and visual neocortex, M1, S1, and V1 in P7 brains were obtained by outlining the corresponding regions with specific probes as indicated in the text and taking measurements from images of whole brains as above. The results are presented as the ratio of the M1, S1, and V1 areas relative to the total dorsal surface area of the cortex. Photographs were taken with an Olympus SZX16 stereomicroscope. Measurements were done using the Imaging software Cell®. All quantified data are expressed as mean values± standard deviation (SD). Significance tests were performed using a 2-tailed Student’s t-test; P-values less than 0.05 were regarded as statistically significant. Each experiment was repeated on at least 4 biological samples for each genotype.

RNA Isolation and RT-qPCR

Total RNA was extracted from dorsal telencephalon of E12.5 or 14.5 embryos using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. cDNA was synthesized starting from 1 or 2 µg total RNA. RT-qPCRs were performed using a StepOnePlus Real-Time PCR system (Applied Biosystems), GoTaq qPCR Master Mix (Promega) and a program optimized by the manufacturer. Gene expression normalization and primers validation were performed as previously described (Saulnier et al. 2013). Endogenous Dmrt5 expression in Dmrt5Tg mice was analysed using previously described primers (Fwd 5′-GGCTG CGCGCCAACAGAGG-3′ and Rev 5′-GTGGTCCCGTCGCTGTCG CT-3′) located in exon 1, excluded from the targeting vector, and exon 2, respectively (Saulnier et al. 2013). Transgenic Dmrt5 expression was evaluated using primers located in the GFP coding sequence inserted downstream of Dmrt5 in the targeting vector: Fwd 5′-AGCTAAAGGGCCACAAGTTC-3′, Rev 5′-GACCCTGTTGCTGGAC-3′. Dmrt5 expression in Emx1-Cre cKO embryos was analysed using the following primers located in predicted exon 1: Fwd 5′-TGCTAATGGAGCCCTCCGAG-3′, Rev 5′-GAACCT CCGGACCCCTCTAG-3′ (Genbank XM_006503093.3). Primers for Dmrt3, Dmrt4, Emx2, Lhx2, Pax6
(Saulnier et al. 2013), and Lef1 (Sohn et al. 2012) have been previously described (Saulnier et al. 2013). The other primers used are as follows: Axin2 Fwd 5’-CCGACCTCAAGTGCAAACTC-3’, Axin2 Rev 5’-ACATAGCCGG AACCTACGTG-3’; Bmp4 Fwd 5’-GAGGGATCTTTACCCTTC-3’, Bmp4 Rev 5’-GTTGAAGGAAAAAGCAG-3’ (sequences given by Dr E. Monuki, Irvine); Wnt3a Fwd 5’-CAGGAACCTC TGGAGATCATGC-3′, Wnt3a Rev 5’-CATGGACAAAGGCTGA CTCC-3’. For RT-qPCR analyses, results were normalized to the level of expression in wild-type forebrain. Error bars show SD of 3 independent experiments.

In Utero Electroporation
cDNAs encoding DMRT5 or tdTomato (Genove et al. 2005) were cloned into the pEFX expression vector (Agarwala et al. 2001). The CP was electroporated at E10.5, and brains were collected at E12.5 (Assimacopoulos et al. 2012). Dmrt5- and tdTomato-containing plasmids were electroporated together so that the electroporation site could be imaged by tdTomato fluorescence immediately after collection of the brains. Brains with appropriately positioned dense electroporation sites were selected as previously described (Toyoda et al. 2010). After sectioning, one series of sections was processed with ISH for tdTomato to mark the site of tdTomato/Dmrt5 co-electroporation. This gave a sharper outline of the site than ISH for Dmrt5 itself, given that Dmrt5 is normally expressed throughout the CP at these ages. Other series of sections from the same brain were processed with ISH to show up- or down regulation of genes of interest. More than 10 brains with appropriate electroporation sites were processed to show expression of each gene of interest in the experimental and control (tdTomato electroporation only) conditions.

Western Blotting Analysis
E12.5 dorsal telencephalon samples containing each tissue from 2 embryos were dissolved in RIPA buffer (NaCl 150 mM, NP-40 1%, Sodium deoxycholate 0.5%, SDS 0.1%, Tris 50 mM pH 8) containing phosphatase (Sigma) and protease (Roche) inhibitors. The samples were sonicated 2 times 5 s. The protein concentration was determined by Bradford assay (OD 595 nm). Of note, 15 μg of each samples were fractionated by SDS-PAGE and transferred to an Immobilon-FL membrane (Millipore), which was incubated with anti-GAPDH (Mouse, 1/10 000, Sigma) and anti-DMRT5 (Rabbit, 1/10 000) antibodies. The secondary antibodies used were anti-rabbit (1/15 000, IR Dye 800CW, and Li-COR) and anti-mouse (1/15 000, IR Dye 680 RD, Li-COR). The detection and quantification were performed using Odyssey Fc (Li-COR) with the Image Studio software.

Results
A Normal Cortical Hem but Reduced Cortical Hemisphere Size in Dmrt5 cKO Mice
To determine whether DMRT5 regulates cortical patterning independent of its influence on the cortical hem, Dmrt5Floxed mice (Dmrt5fl/fl) were crossed with Nestin-Cre, Wnt3a-Cre, and Emx1-Cre mice to generate conditional Dmrt5 knockout mice (cKO mice). Key to this study, this set of cKO mice provided both overlapping and complementary patterns of Dmrt5 deletion. By E12.5, little or no DMRT5 was detected by immunofluorescence (IFl) in the CP of Nestin-Cre cKO mice, but strong DMRT5-IFl remained in the hem and developing choroid plexus. By contrast, Wnt3a-Cre cKO mice, which initiate recombination before E10 (Yoshida et al. 2006), lacked DMRT5-IFl in the hem but displayed DMRT5-IFl equivalent to control levels in the CP and choroid plexus (Fig. 1A). In the E12.5 Emx1-Cre cKO mice, DMRT5-IFl was virtually lost in both the hem and CP. Important to this study, deletion of Dmrt5 in the CP began about a day earlier in Emx1-Cre cKO than in Nestin-Cre cKO mice, consistent with previous observations (Tronche et al. 1999; Gorski et al. 2002; Sahara and O’Leary 2009). We found that DMRT5-IFl was already greatly reduced in the Emx1-Cre
cKOCPatE10.5 but remained strong in Nestin-Cre cKO mice. By E12.5, however, efficient Dmrt5 inactivation was evident in the dorsal telencephalon in both Emx1-Cre cKO and Nestin-Cre cKO lines (Fig. 1A,B and Supplementary Fig.1A).

At E12.5, the cerebral hemispheres were reduced in size in Emx1-Cre cKO (−19.3 ± 7.2%, n = 7) and Nestin-Cre cKO mice (−14.2 ± 7.7%, n = 8) (Fig. 1C). The hemispheres in E18.5 and adult Dmrt5 cKO mice showed slightly greater reduction (E18.5 Emx1-Cre cKO: −20.7 ± 4.0%, n = 12; E18.5 Nestin-Cre cKO: −19.9 ± 5.9%, n = 7; adult Emx1-Cre cKO: −28.1 ± 11.2%, n = 3; adult Nestin-Cre cKO: −21.7 ± 8.6%, n = 4). The cortical hemispheres in Emx1-Cre cKO and Nestin-Cre cKO mice were therefore roughly 20% smaller than control hemispheres, a much less severe reduction than observed in constitutively null Dmrt5 mice at E18.5 (Saulnier et al. 2013). The cortical hemispheres in Wnt3a-Cre cKO and control mice were indistinguishable in size at both E12.5 and E18.5 (n = 7 and 10/genotype, respectively) (Fig. 1C).

Dmrt5 constitutively null embryos show dramatic reduction of Wnt gene expression and WNT signaling activity at the cortical hem (Konno et al. 2012; Saulnier et al. 2013). By contrast, RT-qPCR and section ISH revealed that genes encoding components of the WNT pathway were expressed virtually normally in Nestin-Cre, Emx1-Cre, and even Wnt3a-Cre cKO mice, in which Dmrt5 was deleted specifically in the hem (Supplementary Fig. 2). No significant differences were seen between Dmrt5 cKO mice and control animals in expression levels within the hem of Wnt3a, the earliest Wnt gene expressed selectively in this region (Lee et al. 2000), Lef1, a Wnt nuclear effector, Axin2, a highly reliable indicator of canonical WNT activity, and Bmp4 in the choroid plexus and ventral hem (Furuta et al. 1997; Grove et al. 1998; Ma et al. 2002; Muzio et al. 2005). Domains of Lef1 and Axin2 expression appear, however, slightly smaller in Emx1-Cre cKOs compared with control mice, reflecting the strongly reduced hippocampal primordium (Supplementary Fig. 2A, and see below). In summary, Dmrt5 cKO mice have a cortical hem that appears normal by morphology and gene expression, indicating that severe hem defects in the Dmrt5 null are caused by loss of DMRT5 very early in embryogenesis. We next identified cortical deficits in Dmrt5 cKO mice that persisted despite the presence of the hem.
Figure 1. Cerebral hemispheres are reduced in Dmrt5 Emx1-Cre and Nestin-Cre cKO brains but not in Wnt3a-Cre cKO mice. (A, left to right) Coronal sections through E12.5 brains. Normal DMRT5-IFl in control mice; Nestin-Cre cKO retains DMRT5-IFl in the cortical hem and choroid plexus but not in the CP; Emx1-Cre cKO loses DMRTIFl in both the hem and CP and Wnt3a-Cre cKO loses DMRT-IFl only in the hem. CP, choroid plexus; ET, eminentia thalami; CH, cortical hem; N, neocortex. (B) DMRT5-IFl on coronal sections through E10.5 brains. DMRT5-IFl is already reduced in the cortex of Emx1-Cre mice but is near control levels in Nestin-Cre mice. (C) Dorsal views of E12.5 and E18.5 control and Dmrt5 cKO brains. Graphs representing the surface area of Dmrt5 cKO cerebral hemispheres compared to controls are shown on the right (***P < 0.001).
Hippocampal Fields Are Reduced in Emx1-Cre and Nes-Cre but not in Wnt3a-Cre cKO Mice

At E18.5, the hippocampus was markedly reduced in Nestin-Cre cKO and Emx1-Cre cKO mice, but not in Wnt3a-Cre cKO mice (Fig. 2A). At E18.5, no morphological dentate gyrus (DG) could be identified in H&E-stained coronal sections from Nestin-Cre cKO or Emx1-Cre cKO brains (Fig. 3A). Hippocampal field-specific gene expression (Nrp2, Scip/Pou3f1, KA1/Grik4, and Prox1) revealed that hippocampal fields, including the DG, were present but much smaller than normal in Nestin-Cre cKO and Emx1-Cre cKO mice. In Wnt3a-Cre cKO mice, in contrast, the hippocampus and hippocampal fields resembled those in control mice (Fig. 2B). The decrease in hippocampal field size was more severe in Emx1-Cre than Nestin-Cre cKO embryos, but less so than in Dmrt5 null mice in which the hippocampus is almost completely lost (Konno et al. 2012; Saulnier et al. 2013). Thus, the severity of the hippocampal defect correlated with the time of Dmrt5 deletion in the CP given that Emx1-Cre drives recombination about a day earlier than Nestin-Cre. In summary, while signals from the cortical hem are necessary and sufficient for the hippocampus to form (Mangale et al. 2008), DMRT5 is required in the CP, the responsive tissue, for normal hippocampal development.
Figure 2. Hippocampus is reduced after conditional loss of Dmrt5 in the CP. (A) H&E-stained coronal brain sections at E18.5. The hippocampus is reduced in Nestin-Cre cKO and severely diminished in Emx1-Cre cKO mice (compare with control mouse, far left). In Wnt3a-Cre cKO, in which Dmrt5 is lost only from the hem, the hippocampus resembles the control. (B) Coronal sections through E18.5 brains processed by ISH. All hippocampal fields including the DG are present in cKO mice but smaller in Nestin-Cre and Emx1-Cre cKO animals (arrows). In contrast, individual fields in the Wnt3a-Cre cKO resemble those in the control mouse. HC, hippocampal commissure; N, neocortex.

Emx1-Cre cKO but not Nes-Cre cKO Mice Display Changes in Dorsoventral Cortical Patterning

In the absence of the cortical hem, dorsomedial neocortex is reduced and ventrolateral olfactory cortex, or paleocortex, expands, not only proportional to the smaller hemisphere of the hemispherectomy mouse but also in absolute size relative to control mice (Furuta et al. 1997). A similar shift was previously reported in the Dmrt5 constitutively null mouse (Saulnier et al. 2013) and in the present study was observed in Emx1-Cre cKO mice. Nrp2, encoding a neuropilin receptor, is expressed in paleocortex (Chen et al. 1997; Caronia-Brown et al. 2014). In whole-mount brains and cortical tissue sections processed for ISH at E18.5, Nrp2 expression indicated a larger dorsal extent of the olfactory cortex, in absolute size, compared with control and Nestin-Cre cKO mice (Supplementary Fig. 3A,B). In a complementary pattern, expression of Scip/Pou3f1 marked a notably reduced dorsomedial neocortex in the Emx1-Cre cKO mutants (Supplementary Fig. 3C). Expression of CTIP2 in layer V neurons in the neocor-tex continues ventrally into layer II of piriform cortex, thereby forming a broken band of expression. The position of the break in CTIP2 expression in Emx1-Cre cKO mice further indicated the greater dorsal extent of olfactory cortex (Supplementary Fig. 3D). Finally, Lmo3 expression illustrated the expansion at E18.5 and P7 (Supplementary Fig. 3E).

Relatively little is known about the mechanisms that separate neocortex and paleocortex, except that the transcription factor LHX2 is critical (Chou et al. 2009). Our observations suggest that DMRT5 helps position the boundary between the two types of cortex, and, similar to LHX2, is required earlier than E11.5, before substantial cortical neurogenesis.
Figure 3. Extreme reduction of V1 with conditional deletion of Dmrt5 in the CP. (A) Dorsal views of P7 whole-mount brains processed with ISH for Cdh6, Cdh8, or Rorβ to outline neocortical areas. In
Nestin-Cre and Emx1-Cre cKO brains, the cortical hemispheres are smaller than in control brains, and V1 areas appear minute. In control mice, moderate expression of all three genes outline the triangular area of V1; stronger expression of Cdh8 indicates higher order visual areas surrounding V1. (B) As a ratio of surface area to total hemisphere size, V1 in both cKO mice is significantly reduced (**P < 0.001), S1 and M1 are not significantly different from controls, n = number of samples examined. (C) Control and Wnt3a-Cre cKO hemispheres are indistinguishable in overall size and in the sizes of Rorβ-expressing S1, A1, and V1. (D) Schematic representation of the primary M, S, and V area size observed in the neocortex of the different Dmrt5 cKO mice. Reduced V1 size in Nestin-Cre and Emx1-Cre cKOs is indicated by an asterisk.

Caudomedial Neocortical Areas are Reduced in Emx1-Cre and Nestin-Cre cKO Mice but not in Wnt3a-Cre cKOs

In constitutively Dmrt5 null mouse, the caudomedial neocortex is strongly reduced (Konno et al. 2012; Saulnier et al. 2013). The Dmrt5 null mice rarely survive past birth, preventing identification of the neocortical areas involved. Nestin-Cre cKO and Emx1-Cre cKO mice live on, and at the end of the first postnatal week, area boundaries can be identified by the expression patterns of several genes, including Cdh6, Cdh8, Lmo4, and Rorβ (Assimacopoulos et al. 2012). At postnatal day 7 (P7), V1 is distinguished as a domain of moderate Cdh8 expression, surrounded by stronger Cdh8 expression in higher order visual areas. Cdh8 is also expressed in a frontal region that includes the primary motor area (M1) (Fig 3A, Dmrt5fl/fl). Strong to moderate Cdh6 and Rorβ expression demarcates V1, and primary somatosensory (S1) and auditory (A1) areas; weaker Rorβ expression appears in some higher order visual areas (Fig. 3A, Dmrt5fl/fl).

The area of a cortical hemisphere in dorsal view at P7 was about 70% of control size in Nestin-Cre (71.5 ± 5%, n = 5 litters) and Emx1-Cre (71.6 ± 3.3%, n = 5 litters) cKO brains. Wnt3a-Cre cKO hemispheres were the same size as in control brains (102 ± 4%, n = 3 litters). In both Nestin-Cre and Emx1-Cre cKO whole-mount brains, processed for ISH at P7, the caudomedial area V1 was greatly reduced (Fig. 3A). Changes in the surface areas of V1, S1, and M1 were quantified by determining the ratio of each, demarcated by expression of gene expression markers, versus the total hemisphere surface area. In Emx1-Cre cKO and Nestin-Cre cKO mice, the ratio of the area of V1 to the total hemisphere surface area was roughly half that in control mice, confirming a disproportionate reduction, whereas the ratios of M1 and S1 to total surface area in cKO mice were not significantly different from controls (Fig. 3B). In Wnt3a-Cre cKO mice, the area map outlined by Rorβ expression was indistinguishable from that of control mice (Fig. 3C). These observations (summarized in Fig. 3D) suggest that DMRT5 in neocortical progenitor cells regulates the position and size of V1 and presumably other caudomedial neocortical areas. The smaller overall size of the cortical hemispheres in Emx1-Cre cKO and Nestin-Cre cKO mice compared with controls, however, raises the alternative possibility that loss of Dmrt5 causes both general and region-specific tissue loss that is more evident in the caudomedial region where Dmrt5 expression is highest.

Overexpression of DMRT5 Expands V1 and Reduces the Size of Areas S1 and M1

Challenging the latter possibility, in Dmrt5Tg/+;Emx1-Cre mice with excess DMRT5 in the CP (Supplementary Fig. 1C,D) neocortical area shifts were observed in hemispheres of normal size. Cortical hemisphere area size in Dmrt5Tg/+;Emx1-Cre mice was 101.5 ± 3.6% of that in Dmrt5Tg/Tg controls (n = 5 litters assessed, at least 2 mice per genotype/litter). Because Emx1, like Dmrt5, is expressed in the CP in a high caudomedial to low rostrolateral gradient, Dmrt5 was overexpressed in Dmrt5Tg/+;Emx1-Cre mice following its normal gradient. Heterozygous, Dmrt5Tg/+;Emx1-Cre animals were examined because homozygous mice (Dmrt5Tg/Tg; Emx1-Cre) showed early lethality,
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the findings above, the change in Emx2 expression, in this case an increase, only reached significance at E14.5 (Fig. 5B).
Figure 4. Excess DMRT5 changes area size and position in neocortex. (A) Dorsal views of P7 whole-mount brains processed with ISH for genes indicated. Control hemispheres to the left in each pair; heterozygous DmrtSTg hemispheres to the right. Hemispheres expressing excess Dmrt5 maintain normal overall size but V1 is enlarged. (B) Histograms show that as a ratio of surface area to total hemisphere size, S1, and M1 are significantly reduced (**P < 0.01, ***P < 0.001). n = number of samples examined. (C) Schematic representation of the changes in primary M, S, and V area size observed in the neocortex of Dmrt5 heterozygous Dmrt5Tg mice. Asterisks mark those areas that change in the Dmrt5Tg mouse.

The consequence of Dmrt5 overexpression was also evaluated by IUME into the lateral neuroepithelium of wild-type CD-1 mice at E10.5. Two days after IUME, ectopic Dmrt5 had increased expression of Emx2 and Lhx2, and decreased Pax6 expression (Fig. 5C). Thus, gain and loss of Dmrt5 induce opposite changes in the expression of Emx2, Lhx2, and Pax6, independent of the status of the hem.

In Wnt3a-Cre cKO mice, a change in Pax6 gene expression in the cortical hem was striking, although consistent with findings described above. In wild-type mice, the hem expresses Dmrt5, but specifically excludes expression of Pax6, which extends through neighboring CP. When Dmrt5 is selectively removed from the hem, the Pax6 gradient in the CP was unaffected, as expected, but now the hem strongly expressed Pax6 (Fig. 5D).
Figure 5. Altered levels of Dmrt5 change expression of genes implicated in area map formation. (A and B) Sagittal sections through E12.5 brains processed with ISH for indicated genes, and plots of gene expression changes measured in the dorsal telencephalon by RT-qPCR. (A) In Nestin-Cre and Emx1-Cre cKO mice, Lhx2 expression is downregulated and Pax6 upregulated (compare Pax6
expression in the caudal part of the cortex indicated by arrowheads). Measured by RT-qPCR, Emx2 is not significantly reduced until E14.5. (B) Excess DMRT5 upregulates expression of Lhx2 and downregulates Pax6 (compare expression levels at arrowheads). As in cKO mice, the change in Emx2 expression as assessed by RT-qPCR reaches significance at E14.5 (\(*P < 0.05, \**P < 0.01, \***P < 0.001\)). (C) Overexpression of Dmrt5 with IUME at E10.5, upregulated Emx2 and Lhx2 expression at E12.5, and downregulated Pax6 (arrowheads). The sites of Dmrt5 electroporation are marked by ISH for coelectroporated tdTomato. (D) High magnification views of the hem in E12.5 control and Wnt3a-Cre cKO embryos in coronal sections processed with IFI for DMRT5 and PAX6. In control embryos, the cortical hem shows DMRT5, but not PAX6 IFI; Wnt3a-Cre cKO embryos exhibit ectopic PAX6 IFI in the hem. CH, cortical hem.

Opposite effects on expression of Emx2 and Pax6 by a given manipulation of DMRT5 were predictable, given negative regulation between PAX6 and EMX2 themselves (Muzio et al. 2002). Yet, upregulation of Pax6 expression in the cortex of Emx1-Cre cKO, Nestin-Cre cKO, and Dmrt5 null mice was puzzling given that Lhx2 is required for Pax6 expression (Shetty et al. 2013), and yet is downregulated in these mutants. These observations highlight the complexity of the transcriptional regulation of the Pax6 locus and the importance of DMRT5 in this process.

**Loss of Dmrt3 Results in Reduction of Caudomedial Neocortical Areas**

Like Dmrt5, the related gene Dmrt3 is expressed in cortical progenitors in a high caudomedial to low rostrolateral gradient (Konno et al. 2012) suggesting that Dmrt3 may also contribute to cortical patterning. To test this possibility, we generated Dmrt3 constitutive null mice. Cerebral hemispheres and the hippocampus of Dmrt3 mutants were decreased in size compared with control mice (hemisphere reduction: 17.7 ± 7.2%, n = 7), the reduction observed being less severe than in Dmrt5 null mice (Fig. 6 and Supplementary Fig. 5). At the cortical hem, expression of Wnt and Bmp genes and WNT signaling activity was reduced but much less severely than in Dmrt5 null mice (Fig. 6A). Expression of Emx2 was downregulated, again much less drastically than in Dmrt5 null mice. Pax6 was upregulated and its expression spread medially in the cortex of Dmrt3−/− embryos, but again less strongly than in Dmrt5−/− embryos. Pax6 was conversely downregulated upon Dmrt3 overexpression by in utero electroporation, as observed with Dmrt5 (Fig. 6A,B). Thus, Dmrt3 causes a similar, albeit milder, phenotype to that of Dmrt5 when constitutively deleted or overexpressed.

Dmrt3 null mice survive birth, so that distinct neocortical areas could be examined postnatally with the gene expression markers used above. In P7 whole-mount brains and sagittal sections processed for ISH, V1 appeared smaller in size (Fig. 6C and Supplementary Fig. 5). Based on the dorsal view of the whole-mount images, the ratio of V1 areas to total hemisphere size was estimated to be about 22% reduced (Fig. 6D,E). These observations are consistent with a role for Dmrt3 in regulating the neocortical area map. As for Dmrt5, this hypothesis will require future testing in a mouse line with gain of Dmrt3 function.

**Dmrt5 Expression is Negatively Regulated by DMRT5 and DMRT3**

Given the coexpression of Dmrt3 and Dmrt5 in the same progenitor cells and in matching expression gradients (Konno et al. 2012), combined with both the similarities and differences in the patterning defects in Dmrt3 and Dmrt5 null mice, we asked whether DMRT3 and DMRT5 interact to regulate their gene expression. We assessed Dmrt5 expression levels in Dmrt3 constitutive null embryos and Dmrt3 expression levels in Dmrt5 mutants with ISH and RT-qPCR. The level of Dmrt3 was reduced in Dmrt5 null KO mice and that of Dmrt5 increased in Dmrt3 null KO mice (Fig. 7 and data not shown), demonstrating that DMRT5 upregulates Dmrt3 expression, whereas DMRT3 downregulates...
expression of Dmrt5. A third member of the Dmrt gene family, Dmrt4 (Dmrt4a), is expressed in an opposite gradient to Dmrt3/5 and is positively regulated by PAX6 (Kikkawa et al. 2013). Given the opposing gene expression patterns, upregulation of Dmrt4 expression in Dmrt3 and Dmrt5 null mutant mice was as predicted (Fig. 7 and data not shown).

Expression of Dmrt5 is not only downregulated by DMRT3 but also by DMRT5 itself. In E12.5 Dmrt5Tg/Tg; Emx1-Cre mice, a higher level of total DMRT5 protein, compared with control mice, was as expected evident with DMRT5-IF, and western blotting. In heterozygous Dmrt5Tg/+; Emx1-Cre mice, the level of DMRT5 detected appeared intermediate between levels in homozygous Dmrt5Tg/Tg; Emx1-Cre mice and controls. In western blots, DMRT5 levels were estimated in heterozygous and homozygous Dmrt5Tg mice, respectively, as 1.8- and 2.6-fold times the level in controls (Fig. 8A,B). Surprisingly, ISH and RT-qPCR assays revealed that while the level of transgenic Dmrt5 transcript, reflected by Dmrt5-IRES-linked eGFP expression, was increased in the cortex of heterozygous and homozygous Dmrt5Tg mice, the level of endogenous Dmrt5 transcript was diminished (Fig. 8C).

Conversely, the level of Dmrt5 exon 2 deleted transcripts was increased in the cortical epithelium of Emx1-Cre cKO mice (Fig. 8D). These findings suggest that Dmrt5 mRNA level is tightly controlled in a negative feedback loop by DMRT5 itself and DMRT3 (Fig. 8E). Because abnormal levels of Dmrt genes cause marked aberrations in cortical patterning, including the neocortical area map, such fine control of their expression is consistent with a critical role in normal cortical development.

Discussion

Dmrt5 is Directly Required for Several Processes in Cortical Patterning

Constitutive loss of Dmrt5 causes the hippocampus and caudomedial neocortex to shrink, and the boundary of paleocortex to shift dorsally, strongly resembling cortical changes in hem-ablated mice (Konno et al. 2012; Saulnier et al. 2013; Caronia-Brown et al. 2014). Given that constitutive deletion of Dmrt5 virtually obliterates the hem, a ready conclusion is that Dmrt5 has its effects on cortex because of the loss of hem WNT and BMP signals. In the present study, however, Dmrt5 was conditionally deleted from cortical progenitors after the hem had formed. No overt defects were seen in the hem with respect to its size, morphology, or expression of a variety of genes associated with WNT or BMP signaling. Nonetheless, abnormalities in the hippocampus and neocortex, and at the boundary between neocortex and paleocortex, were similar to those observed in constitutive Dmrt5 null mice. These findings indicate that Dmrt5 has several important functions in forebrain development, first establishing the hem, second acting in cortical progenitor cells to direct functional cortical subdivisions, and third contributing to the correct formation of the neocortical area map.

A parsimonious interpretation is that DMRT5 loss in the CP and consequent influences on Dmrt downstream effector genes are also responsible for the cortical defects in the constitutively Dmrt5 null mouse. The defects are more severe in the null than in cKO mice because Dmrt5 was deleted earlier in development. This interpretation is supported by the greater severity of cortical defects in Emx1-Cre cKO mice than in Nestin-Cre cKO animals, given that Emx1-Cre drives gene deletion in the CP about a day before Nestin-Cre (Gorski et al. 2002; Sahara and O’Leary 2009; Shetty et al. 2013). Thus, alteration in the position of the neocortical/paleocortical boundary was observed in Emx1-Cre but not in Nestin-Cre cKO mice, suggesting that DMRT5, like LHX2 (Chou et al. 2009) regulates this boundary before E11.5 and the start of substantial cortical neurogenesis. Additionally, a reduced
hippo-campus was observed in both cKO mice, but the reduction was more severe in Emx1-Cre than in Nestin-Cre cKO mice.
Figure 6. V1 reduced in Dmrt3-/− mice with modest reduction of cortical size and hem gene expression. (A) RT-qPCR analysis of the expression of the indicated genes in the cortex of Dmrt3-/− mice and, for comparison, in Dmrt5-/− mice (*P < 0.05, **P < 0.01, ***P < 0.001). (B) Coronal brain sections of E12.5 embryos. Expression of Emx2 is decreased in Dmrt3-/− embryos (asterisk) and Pax6 is increased (see asterisks). IUME of Dmrt3 downregulates Pax6 expression (arrow). (C) Dorsal views of P7 whole-mount brains processed by ISH for the indicated genes. (D) Graphs representing the overall change in hemisphere size in Dmrt3 null mice, and the disproportionate reduction of V1 (**P < 0.01, ***P < 0.001). (E) Schematic representation of the changes in primary M, S, and V area size observed in the neocortex of the Dmrt3 cKO mice.
DMRT5 Controls the Size and Position of Neocortical Areas

Both the constitutive null Dmrt5 mutant (Saulnier et al. 2013) and cKO Dmrt5 mutants have smaller cortical hemispheres than wild-type mice, indicating an important function for Dmrt5 in growth control, which remains to be fully characterized. Because Dmrt5 expression is higher caudomedially, loss of Dmrt5 might cause the notably disproportionate reduction in the growth of caudomedial cortex. We therefore determined the boundaries of cortical regions and the size and position of neocortical areas in mice conditionally overproducing DMRT5 (Dmrt5Tg mice), which had normally sized cortical hemispheres. In Dmrt5Tg mice, excess DMRT5 protein was generated throughout the CP, but did not enlarge the hippocampus, or the overall surface area of the neocortex. In this context, V1 was roughly half larger in heterozygous Dmrt5Tg mice than in control mice, and M1 and S1 were smaller. That is, DMRT5 regulated the size and position of areas in a manner corresponding to the caudomedial high to rostrolateral low expression gradient of Dmrt5, retained in the Dmrt5Tg/+; Emx1-Cre mice. These findings are hard to square with the hypothesis that DMRT5’s effects on tissue growth caused the neocortical area changes, given that DMRT5 was also available in excess at the sites of M1 and S1 generation, yet these areas were reduced. Also inconsistent with a model in which DMRT5 regulates regional boundaries in cortex by promoting region-specific tissue interactions are the findings that the size and position of V1 were increased in the Dmrt5Tg mice, while M1 and S1 were reduced, suggesting a role for DMRT5 in regulating the size and position of neocortical areas in a manner consistent with the caudomedial high to rostrolateral low expression gradient of Dmrt5.
growth is the observation that ventrolateral cortex, where Dmrt5 expression is lowest, enlarges with deletion of Dmrt5.

**Interactions Between DMRT5 and Other Known Cortical Transcription Factors**

DMRT5 regulates the expression of several transcription factors whose roles in patterning the cerebral cortex have been more thoroughly explored. They include EMX2 (Hamasaki et al. 2004; Zembrzycki et al. 2015), PAX6 (Bishop 2000; Bishop et al. 2002; Backman et al. 2005; Zembrzycki et al. 2013), and LHX2 (Mangale et al. 2008; Chou et al. 2009; Shetty et al. 2013). Findings from the present study suggest that DMRT5 both interacts with these transcription factors in cortical patterning and functions independently.

Similar to excess DMRT5, excess EMX2 generates a neocortex with an abnormally large V1 and correspondingly smaller S1 and M1 (Hamasaki et al. 2004); thus, the relationship between Emx2 and Dmrt5 is particularly relevant. Dmrt5 expression is unaffected in mice lacking Emx2, whereas Emx2 expression is downregulated in Dmrt5 null mice, indicating that Emx2 lies downstream of Dmrt5 (Saulnier et al. 2013). The most dramatic effects of manipulating DMRT5 levels on the neocortical area map, however, seem likely to reflect an activity of DMRT5 independent of EMX2. Excess DMRT5 in the CP from E10.5 caused extensive changes in the neocortical area map, but only a modest and relatively late (E14.5) upregulation of Emx2 expression. We suggest that in wild-type mice DMRT5 acts directly on the neocortex but in concert with EMX2. Such a cooperation would explain a previous finding, namely that decreasing the excess FGF8 observed in Emx2 null mice rescues at least part of the Emx2 mutant cortical phenotype (Fukuchi-Shimogori and Grove 2003). Excess FGF8 would also downregulate Dmrt5 expression, so that reducing the FGF8 surplus would mitigate a joint Emx2/Dmrt5 phenotype.

Excess DMRT5 reduces expression of Pax6, which could also contribute to the neocortical phenotype observed in Dmrt5Tg/+; Emx1-Cre mice. Reduced rostral and expanded caudal areas have been reported in mice constitutively lacking Pax6 but because these mice die at birth, neocortical areas could not be definitively identified with postnatal gene expression patterns (Bishop 2000; Bishop et al. 2002). Conditional deletion of Pax6 in cortical progenitors, utilizing the same Emx1-IRES-Cre used in the present study (Gorski et al. 2002), halves the size of the cortical hemispheres and hugely diminishes the absolute size of S1 with no overt disproportional changes in the size of either V1 or frontal cortex (Zembrzycki et al. 2013). Reducing Pax6 expression in Dmrt5Tg/+;Emx1-Cre mice might therefore contribute to a smaller S1, but not to the greatly enlarged V1. That Dmrt5Tg/+;Emx1-Cre cortical hemispheres were indistinguishable in size from wild-type mice is further generally inconsistent with a role for Pax6 reduction in the Dmrt5Tg/+;Emx1-Cre phenotype.

DMRT5 upregulates expression of Lhx2, and in Emx1-Cre Dmrt5 cKO mice Lhx2 expression is reduced by about half (Fig. 7A,C). With conditional deletion of Lhx2 at E10.5, utilizing the same Emx1-Cre driver line as in the present study (Gorski et al. 2002), neocortical progenitors are re-fated to generate neurons for paleocortex (Chou et al. 2009). Heterozygous Lhx2 cKO mutants, however, do not show this phenotype (Chou et al. 2009). In the Emx1-Cre Dmrt5 cKO mice, a shift in the boundary between neocortex and paleocortex is observed, much more modest than in the Emx1-Cre Lhx2 cKOs. Thus, halving the levels of Lhx2 could potentially contribute to this defect. Gli3 is another transcription factor involved in regulating the separation between the neocortex and paleocortex.
Figure 8. Negative autoregulation by Dmrt5. (A) Sagittal (top) and coronal (bottom) brain sections processed by IFI for DMRT5 on E12.5 control (Dmrt5Tg/Tg), Dmrt5Tg/+; Emx1-Cre, and Dmrt5Tg/Tg;Emx1-Cre embryos. DMRT5-IFI is increased and more uniform in Dmrt5Tg embryos. (B) Western blot shows a higher level of DMRT5 protein in the dorsal telencephalon of E12.5 Dmrt5Tg/+;Emx1-Cre and Dmrt5Tg/Tg;Emx1-Cre mice than in control non-Cre-excised Dmrt5Tg/Tg embryos. Dmrt5-/- embryos were used as negative controls. Bar graph shows quantification of three distinct western blots. For each sample, the intensity of the Dmrt5 band was divided by the intensity of the GAPDH band to account for loading differences. Expression levels in the Dmrt5Tg/+;Emx1-Cre
and Dmrt5Tg/Tg;Emx1-Cre embryos were calculated relative to the controls (Dmrt5Tg/Tg), which was assigned a value of 1 (*P < 0.05, **P < 0.01, ***P < 0.001). (C and D) ISH for Dmrt5, E12.5 coronal brain sections (top); quantitative RT-qPCR analysis of Dmrt5 (bottom) in the dorsal telencephalon of E12.5 embryos. Endogenous Dmrt5 transcripts are reduced in Dmrt5Tg/+;Emx1-Cre and Dmrt5Tg/Tg;Emx1-Cre embryos and upregulated in Emx1-Cre cKO (*P < 0.05, **P < 0.01, ***P < 0.001). Dmrt5Tg/Tg or Dmrt5fl/fl were used as controls. Transgenic Dmrt5 expression in Dmrt5Tg/+;Emx1-Cre and Dmrt5Tg/Tg;Emx1-Cre embryos was evaluated using GFP primers. (E) Model of Dmrt5 and Dmrt3 gene action in the CP downstream of cortical hem signals based on results from this work and others (Konno et al. 2012; Saulnier et al. 2013).

(Amaniti et al. 2015). Its expression is largely unaffected in Dmrt5−/− and Dmrt3−/− mutants (Saulnier et al. 2013). Our results allow us to consider only limited potential transcription factor interactions. Functional interactions among Dmrt5, Emx2, Pax6, and Lhx2 with respect to cortical subdivision and formation of the neocortical area map require further investigation. Direct transcriptional regulation between members of this group, as well as overlap in their transcriptional targets, also remain to be determined. Genes that control patterning of cerebral cortex and the formation of the neocortical area map are also tightly involved in the control of the proliferation/differentiation of cortical progenitors. Several studies have associated Dmrt with proneural genes (Huang et al. 2005; Yoshizawa et al. 2011). Further functions of the DMRT subgroup in regulating proneural genes, and in neurogenesis more generally, remains to be explored.

DMRT3 also Contributes to the Control of Cortical Development

Dmrt genes have been studied extensively for their roles in sex determination and sexual differentiation, conserved widely across metazoans (Hong et al. 2007; Bellefroid et al. 2013). Recently, Dmrt genes have been found to have functions in neural development, including neuronal specification in the vertebrate spinal cord (Andersson et al. 2012). Dmrt3, Dmrt4, and Dmrt5 belong to a Dmrt gene subfamily that contains a DMA domain. All three are expressed in gradients in the CP, and their functions and interactions in cortical development have yet to be fully investigated. Our analysis of the phenotype of Dmrt3 constitutively null mice suggests that Dmrt3 has a similar function to Dmrt5 in cortical development, including a role in generating the neocortical area map. The importance of Dmrt3 in cortical development inferred from the phenotype of Dmrt3 null KO mice is likely to be underevaluated given the fact that the absence of Dmrt3 may be compensated by the increase of Dmrt5.

How Dmrt3 functions with Dmrt5 to control cortical patterning is unclear. Dmrt3 mRNA is reduced in Dmrt5 constitutive null mice suggesting that Dmrt3 may function downstream of Dmrt5. Whether they act in concert and whether they directly or indirectly regulate Wnt, Bmp, Emx2, Lhx2, or Pax6 gene expression remain to be addressed. Finally, the possible role of Dmrt4 in cortical patterning remains to be considered.

Negative Autoregulation of Dmrt5

Negative feedback is an important component of many biological networks. Computational and experimental approaches have shown that genes regulated by negative feedback have more stable expression than other genes (Hasty et al. 2002). Our identification of negative feedback in Dmrt5 regulation in the developing telencephalon highlights the probable importance of maintaining appropriate levels of its expression in the fast growing telencephalic vesicles during embryonic development.
Our evidence for negative autoregulation comes from the finding that endogenous Dmrt5 mRNA levels are reduced in Dmrt5Tg mice and that Dmrt5 exon 2 deleted transcript levels are increased in Emx1-Cre Dmrt5 cKO mice. Despite the reduction of endogenous Dmrt5 mRNA level in Dmrt5-overexpressing Dmrt5Tg mice, total DMRT5 protein levels were elevated, presumably because of the highly efficient transcription of the Dmrt5 transgene at the ROSA26 locus. We cannot exclude that other mechanisms such as posttranscriptional ones may also participate in the elevation of DMRT5 protein levels observed in the Dmrt5Tg mice. In transgenic mice overexpressing the human PAX6 gene, elevated PAX6 protein levels are observed despite negative feedback autoregulation of PAX6 expression (Manuel et al. 2007).

The negative transcriptional autoregulation of Dmrt5 may be direct, as in the case of Pax6 autoregulation (Aota et al. 2003; Kleinjan et al. 2004), or indirect given that DMRT5 influences the expression of many other transcription factors (Fig. 5). Autoregulation has been demonstrated, however, for another member of the Dmrt gene family, Dmrt1, which is critical for testis development (Murphy et al. 2010). Negative regulation of Dmrt5 further involves DMRT3, given that Dmrt5 mRNA is increased in the Dmrt3 null KO. While the mechanisms controlling Dmrt5 expression are likely to be complex and remain to be investigated, our findings implicate negative autoregulation as an important stabilizing component of DMRT5 protein level in the developing cortical neuroepithelium.

**Supplementary Material**

Supplementary material is available at Cerebral Cortex online.

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**References**


